



## Installation Restoration Research Program

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# **Microbial Requirements for In Situ Biotreatment of Explosives**

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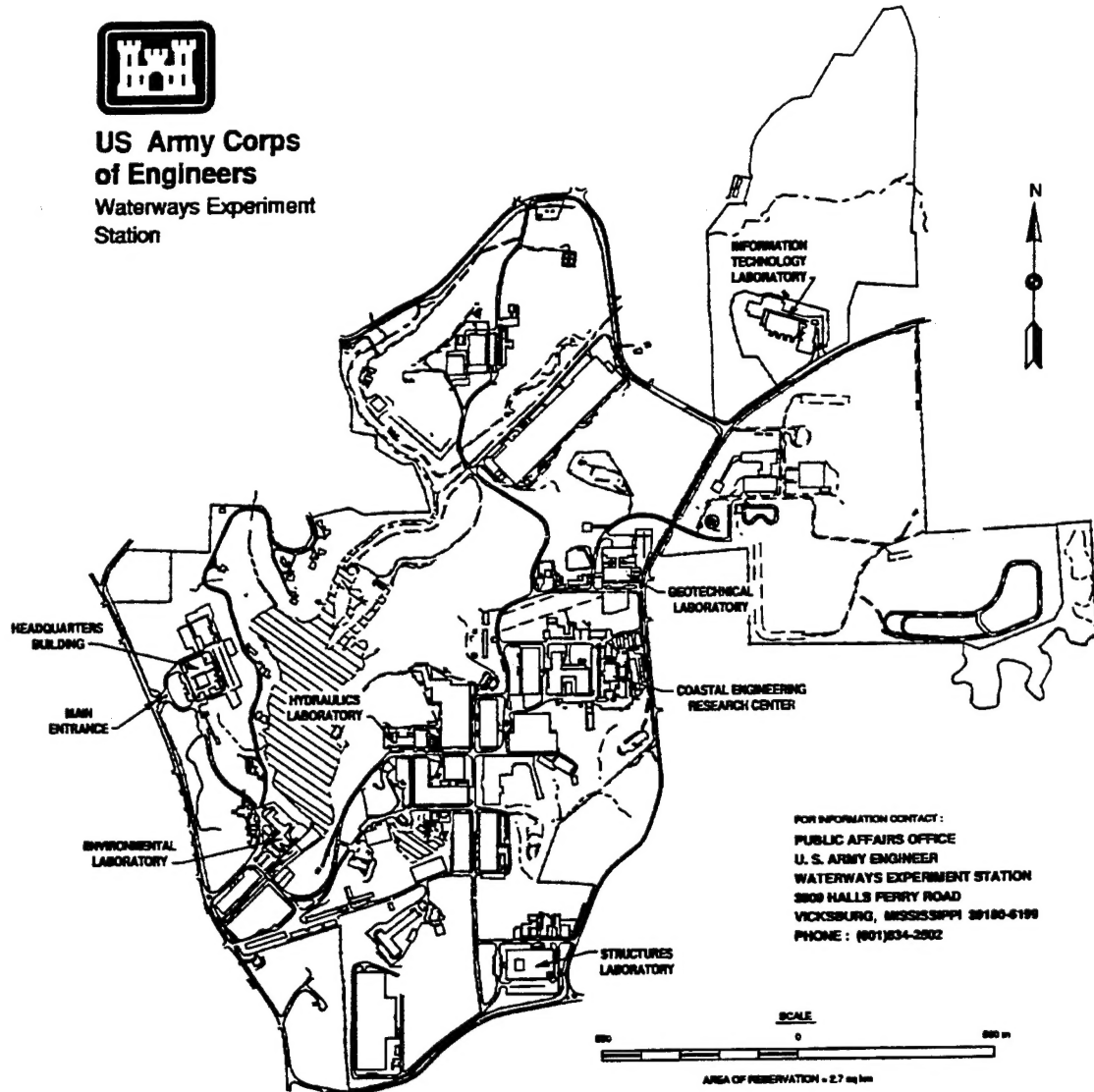
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# Preface

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This report was prepared by the Environmental Laboratory (EL) of the U.S. Army Engineer Waterways Experiment Station (WES) as part of the Installation Restoration Research Program (IRRP). The research was funded by the Environmental Quality Technology Program, Work Unit AF25-ET-004. The program is managed by Dr. M. John Cullinane, EL. MAJ Kevin Keehan was the Technical Monitor for the U.S. Army Environmental Center. Mr. Richard Waples was the Technical Monitor for the U.S. Army Corps of Engineers Military Programs.

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At the time of publication of this report, Dr. Robert W. Whalin was Director of WES. COL Bruce K. Howard, EN, was Commander.

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# 1 Introduction

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## Background and Relevance

Degradation of organic contaminants into basic inorganic components (e.g., carbon dioxide, water, and nitrate) is termed *mineralization*. Earlier work by several investigators indicated that 2,4,6-trinitrotoluene (TNT) can be biologically transformed into several organic by-products, some of which are more toxic than TNT (Carpenter et al. 1978; McCormick, Feeherry, and Levinson 1976; Kaplan and Kaplan 1982). For example, Kaplan and Kaplan (1982) identified 2-amino-4,6-dinitrotoluene (2ADNT), 4-amino-2,6-dinitrotoluene (2ADNT), 2,4-diamino-6-nitrotoluene (2,4DA6NT), 2,6-diamino-4-nitrotoluene (2,6DA4NT), 2',4,6',6-tetranitro-2,4'-azoxytoluene, 2,2',6,6'-tetranitro-4,4'-azoxytoluene as biotransformation products formed under aerobic, organically rich conditions. Recent research, such as that of Funk et al. (1993), Boopathy and Kulpa (1992), Preuss et al. (1993), Duque et al. (1993), as well as emerging research including that of Crawford (1995) and Funk et al. (in press), indicate that mineralization of TNT is possible.

However, despite these findings, high levels of TNT persist in the soils of many military installations. Remediation of soils contaminated with TNT is a serious problem for military installations. Bioremediation with TNT-mineralizing microorganisms is a potentially cost-effective technology with several possible variations, including composting, bioslurry, land-farming, and in situ treatment. In situ biotreatment is an emerging technology for the remediation of both saturated and unsaturated soils (Sims et al. 1993). This technology has been widely applied for the treatment of petroleum hydrocarbon contamination and, to a lesser extent, chlorinated solvents. However, in situ biotreatment has had limited utility for the treatment of TNT-contaminated soils because this compound tends to remain in the surface layer and is therefore more concentrated and more toxic. Bioslurry treatment has the potential to be rapid and highly effective because of the intimate contact between the contaminated soil, TNT-degrading microorganisms, dissolved oxygen, water, and activity-enhancing chemicals that may be added (Zappi et al. 1992a). However, the bioslurry treatment process requires excavation and handling of contaminated soils and the addition of energy for stirring and/or heating. For these reasons, the use of bioslurry treatment is probably best restricted to highly contaminated soils for which the use of in-place treatment

techniques is unsuitable due to the prolonged treatment time required for contaminant removal. Land treatment is highly desirable for explosives because it minimizes soil excavation and lowers energy requirements for remediation.

## Test Rationale

A three-tiered approach to determine the microbial requirements for land-farming biotreatment of explosives was developed (Figure 1). The tiered approach requires an initial soil characterization followed by Tier I, the screening plate test developed previously (Gunnison et al. 1993). This test is based on the addition of TNT with and without the use of additional chemicals added to separate treatments to stimulate microbial activity. Chemical treatments include toluene, dinitroaniline, dinitro-o-cresol, dinitrophenol, sodium acetate, sawdust, or sodium succinate. Following 1 to 4 weeks of incubation in static and slurry modes, individual soil treatments are plated onto a crystal-line lawn of TNT overlying a basal salts agar containing one of three cosubstrates (sodium acetate, glucose, or sodium succinate). Activity against TNT is detected by visual observation of TNT clearing around the soil sample. The procedure is used to determine the presence of native microorganisms active against TNT in the soil slated for treatment and the cosubstrate(s) required to support degradation. If microorganisms capable of degrading the contaminant(s) are not present, then nonbiological clean up alternatives should be considered. If effective microorganisms are present, Tier II shake flask tests are conducted.

Laboratory studies of TNT biodegradation have demonstrated that TNT concentration can be extremely variable. Extractable TNT levels can be distributed very evenly in a soil sample that has been dried, ground, and thoroughly mixed. However, once the sample has been divided into replicates and each replicate has been moistened, heterogeneous abiotic and microbial activities and sorptive processes produce a varied distribution of TNT and its transformation products. Therefore, replicates for each sample interval are required to minimize sample variation.

In Tier II, combinations of nutrients, cosubstrates, and/or surfactants required to maximize the removal of TNT, while minimizing the production of undesirable products, are determined. The use of shake flask testing was advocated by the in situ biotreatment advisory committee (Zappi et al. 1992a), which indicated that this is the best procedure for assessing the degradability of comparatively recalcitrant (difficult to biodegrade) compounds. As with soil bioslurry treatment, shake flask conditions provide optimum contact of the degrading biomass with moisture, nutrients, cosubstrates, surfactants, and the contaminated soil. If recalcitrant compounds cannot be degraded under these conditions, little chance exists that they will be degraded in static soils having lower moisture content and less intensive liquid-to-solids contact. By contrast, combinations of ingredients found to be effective under shake flask conditions

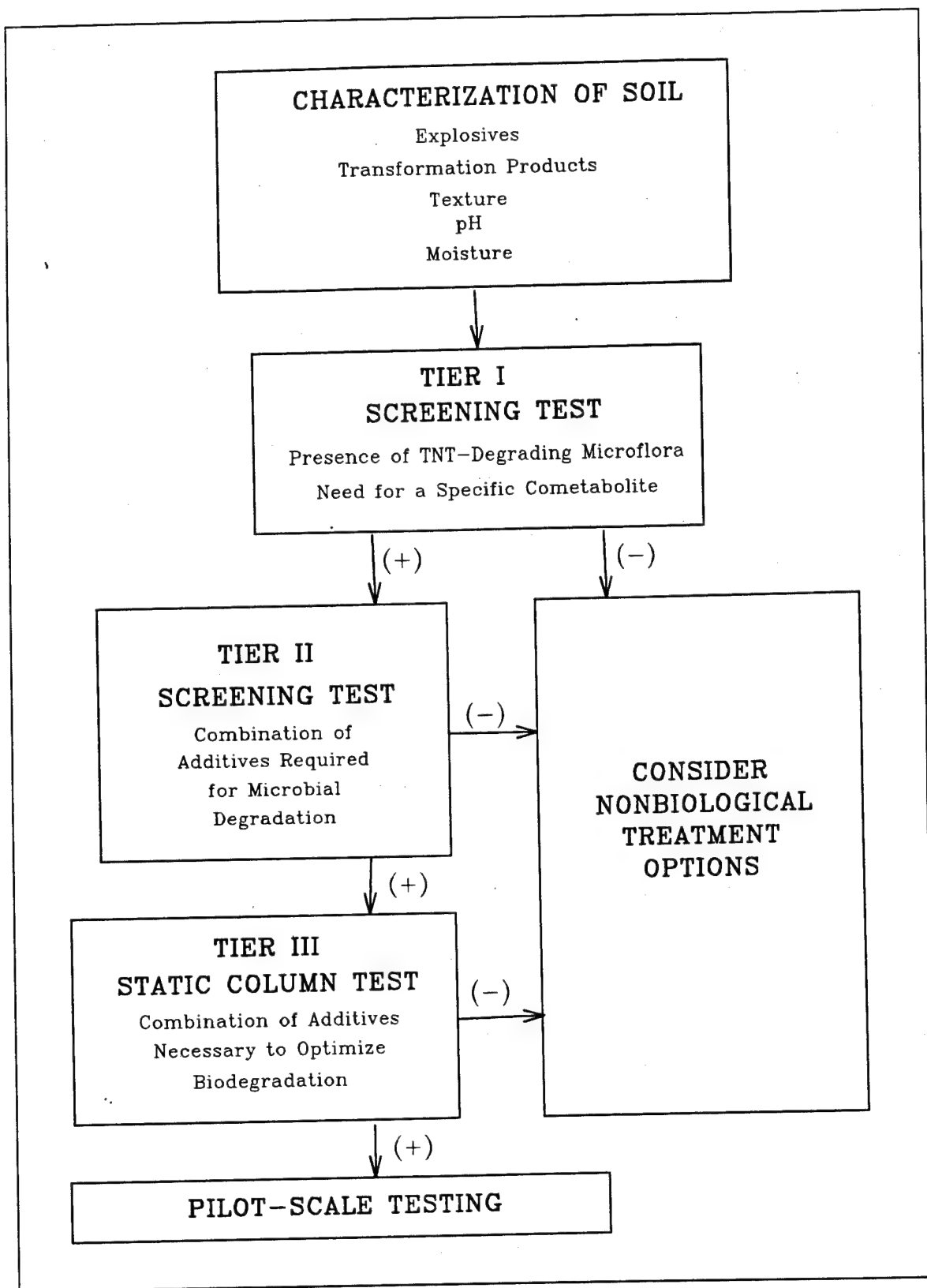


Figure 1. Three-tiered system to evaluate microbial requirements to support degradation of TNT during land-farming biotreatment of TNT-contaminated soils

may be effective in facilitating the biotreatment of surface soils in a static test. A main goal in developing the test was to keep the execution as simple as possible; i.e., a load-and-forget system that can be examined at the start and at the end of a reasonable incubation period. If results of Tier II tests indicate that biodegradation rates are unacceptably low, a nonbiological treatment alternative should be considered for the soil. However, if an acceptable degradation rate is achieved, Tier III Static Cell tests are conducted.

In Tier III, treatment effectiveness is evaluated under simulated field conditions in static cells. The cell tests the ability of microflora to biologically destroy TNT when the microflora is supplied with the nutrients, cosubstrates, and/or surfactants (additives) under simulated field conditions. For Tier II, the application of additives with a sprayer simulates periodic addition directly onto the surface or intermittent incorporation by tilling. Therefore, the treatments allow a downward movement of moisture and nutrients, permitting complete saturation of the soil, if necessary. The test apparatus is large enough to permit the removal of 3 to 5 replicates of each treatment for every sampling interval. Since degradation is anticipated to occur very slowly, treatment periods of several weeks to several months are necessary. The system also permits incubation of the treatments in the dark to prevent photolytic degradation of TNT. Ventilation prevents the stagnation of air or the accumulation of excessively high moisture content. Individual treatments are easily accessible for sampling, addition of nutrients, and monitoring of moisture and soil gas content. The system, described in the static cell test procedure section below, consists of an incubation chamber containing 40 individual test units, or cells, on sliding trays.

If the degradation rate is suitable in Tier II, the compliment of additives and the optimal conditions generated in Tier III can be used to design pilot-level tests.

## Objectives

To obtain cost-effective remediation of explosives-contaminated soils, effective treatment technologies must enhance degradation pathways of endogenous microflora within the contaminated soil matrix. This work was undertaken to develop simple tests to determine the chemical compounds (additives) required to stimulate TNT destruction by site-specific native microflora and to assess the feasibility of obtaining treatment in static soil, where surface application must be used to distribute degradation activity-enhancing additives.

Specific objectives were to rapidly determine which additives (nutrients, surfactants, cosubstrates) are required to support rapid, extensive mineralization of TNT by native microbial populations at contaminated sites and to determine which combination of additives achieves maximum destruction of TNT under static soil conditions simulating surface application in the field.

## 2 Methods and Materials

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### Soil Selection

Four candidate soils for shake test development were selected based on their history of TNT contamination (soils 1 through 4, Table 1). The Hastings East Industrial Park (HEIP) soil, also listed in Table 1, was used for the static soil test development because previous biotreatment experiments demonstrated extensive TNT destruction (Gunnison et al. 1993, Zappi et al. in press).

<b>Table 1</b> <b>Sources of Soils Used in This Study</b>				
Soil Number	Sampling Location	Particle Size Composition, %		
		Sand	Silt	Clay
1	Seattle, WA	77.5	22.5	0
2	Crane, IN	19.0	58.0	23.0
2	Hastings, NE	33.0	54.0	13.0
4	McAlester, OK	42.5	42.5	15.0
5	WES reference soil	0	90.0	10.0

### Soil Handling

Surface soils obtained from each of the sites were sieved through 0.5-cm mesh netting to remove rocks and large chunks of other materials and were stored at 4 °C until used. Samples of these soils were analyzed to determine particle size, organic matter content, and TNT and TNT transformation products. Dry weight was assessed by weight loss of 10 g of soil after drying for 12 hr at 105 °C. Soils were tested for microbial activity against TNT using the methods of Gunnison et al. (1993).

Soils were dried for 2 to 5 days at room temperature, placed into two 5-gal<sup>1</sup> carboys and then mixed on a roller mill at 4 to 5 rpm for 24 hr. Soils from each carboy were combined in a separate 5-gal carboy, mixed, and then placed back into the original carboys for an additional 24 hr of mixing. The soil was then passed through a 2-mm sieve, placed into a screw-capped plastic bottle, and refrigerated until used.

## Shake Test Procedure

### Shake flask microcosms

A 12-g dry weight equivalent of soil (DWE) and 25 mL of medium were added to each test flask. High nutrient media consisted of 46.5 mg NH<sub>4</sub>Cl and 23.8 mg K<sub>2</sub>HPO<sub>4</sub> per liter of reverse osmosis (RO) water. Low nutrient treatments received one-tenth of the high nutrient treatment. Cosubstrates differed from soil to soil and were selected based on the one or two cosubstrates producing the strongest positive results in the screening test (Gunnison et al. 1993). These cosubstrates included one or both of the following: 1 percent acetate or 0.1 percent toluene. All samples were prepared in triplicate and incubated for 30 days with shaking on a gyratory shaker at 75 rpm and 25 °C.

### Treatment conditions

Treatment conditions included an acidified control killed by the addition of 1 mL of 1M HCl; a biotic control (water, but no nutrients or cosubstrate); low nutrients (ammonium and phosphate) only; low nutrients with Tween 80<sup>TM</sup>; low nutrients plus cosubstrate only; low nutrients plus Tween 80 plus cosubstrate; high nutrients (ammonium and phosphate) only; high nutrients with Tween 80 only; and high nutrients plus Tween 80 plus cosubstrate. When more than one cosubstrate was examined, each was tested individually with the same treatment combinations.

### Sample collection and treatment

Samples were taken initially and after 30 days of incubation with shaking. At the end of incubation, each soil was suspended and aseptically transferred into sterile 150-mL Corex<sup>TM</sup> centrifuge bottles (Corning Glass, Inc., Corning, NY). Shake flasks were rinsed three times with sterile distilled water, and this was added to the soil. The soil was collected by centrifuging at 6,084 × g for 20 min. The supernatant was decanted, and the volume of the supernatant was determined. Then, 5 mL of the supernatant was collected for

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<sup>1</sup> To convert gallons (U.S. liquid) to cubic meters, multiply by 0.003785412.

the analysis of TNT and TNT transformation products. A 1-g subsample of the wet pellet material was taken for each of the following determinations: dry weight, chemical analysis, and microbial plate counts.

## **Static Cell Test Procedure**

### **Establishment of Tween 80 treatment levels**

Dilutions of Tween 80<sup>TM</sup> surfactant in distilled deionized water were prepared containing 0.0, 0.25, 0.50, 0.75, 1.0, 2.0, 3.0, 5.0, and 10 percent (v/v) concentrations of the surfactant. Three replicate 15-mL volumes of each dilution were measured into glass bottles and sealed with teflon-lined screw caps. A large mass (>0.1 g) of crystalline TNT was added to each solution, and each mixture was vortexed for 30 sec. The mixtures were stored in the dark at room temperature until analyzed. A 10 percent Tween 80 control sample without added TNT was also run.

When Hastings soils were treated in a previous slurry study approximately 50 mg/L of TNT desorbed from Hastings soil into the aqueous phase (Gunnison et al. 1993). Based on the assumption that TNT-degrading microorganisms will only utilize TNT in the aqueous phase, as much TNT was introduced into the aqueous phase of the test matrix as possible. The results of this study indicate that a Tween 80 concentration of 1.4 percent (v/v) would maintain TNT solution levels at approximately 150 mg/L (Figure 2).

### **Development and construction of experimental soil test cells**

A test was conducted to determine whether soil test cells should be constructed of teflon or polypropylene due to the potential sorption of TNT and TNT transformation products by the material used. Six 250-mL centrifuge bottles of polypropylene and of teflon were filled with water spiked with a solution containing 2 mg of 2ADNT, 2 mg of 4ADNT, and 6 mg of TNT per liter. The test cells were incubated in the dark under static conditions. At 12 and 34 days, half the bottles of each type were analyzed for 2ADNT, 4ADNT, and TNT.

For unsaturated soil studies, each 250-mL centrifuge bottle was modified to include a moisture probe through the side and a small drain valve at the bottom (Figure 3). During testing using unflooded soil treatments, the valves were normally left open, but no drainage was observed with any of the moisture doses applied. (For flooded soil studies, drain valves will be kept closed and the probes omitted.) The bottom of each cell was lined with a circular disc of geotextile material having a diameter the same as the inside diameter of the cell. A 25-g layer of garnet sand was placed on top of the filter and another geotextile filter of the same diameter was placed on top of the sand. A Watermark<sup>TM</sup> Model 200 moisture probe (Irrometer Company, Riverside,

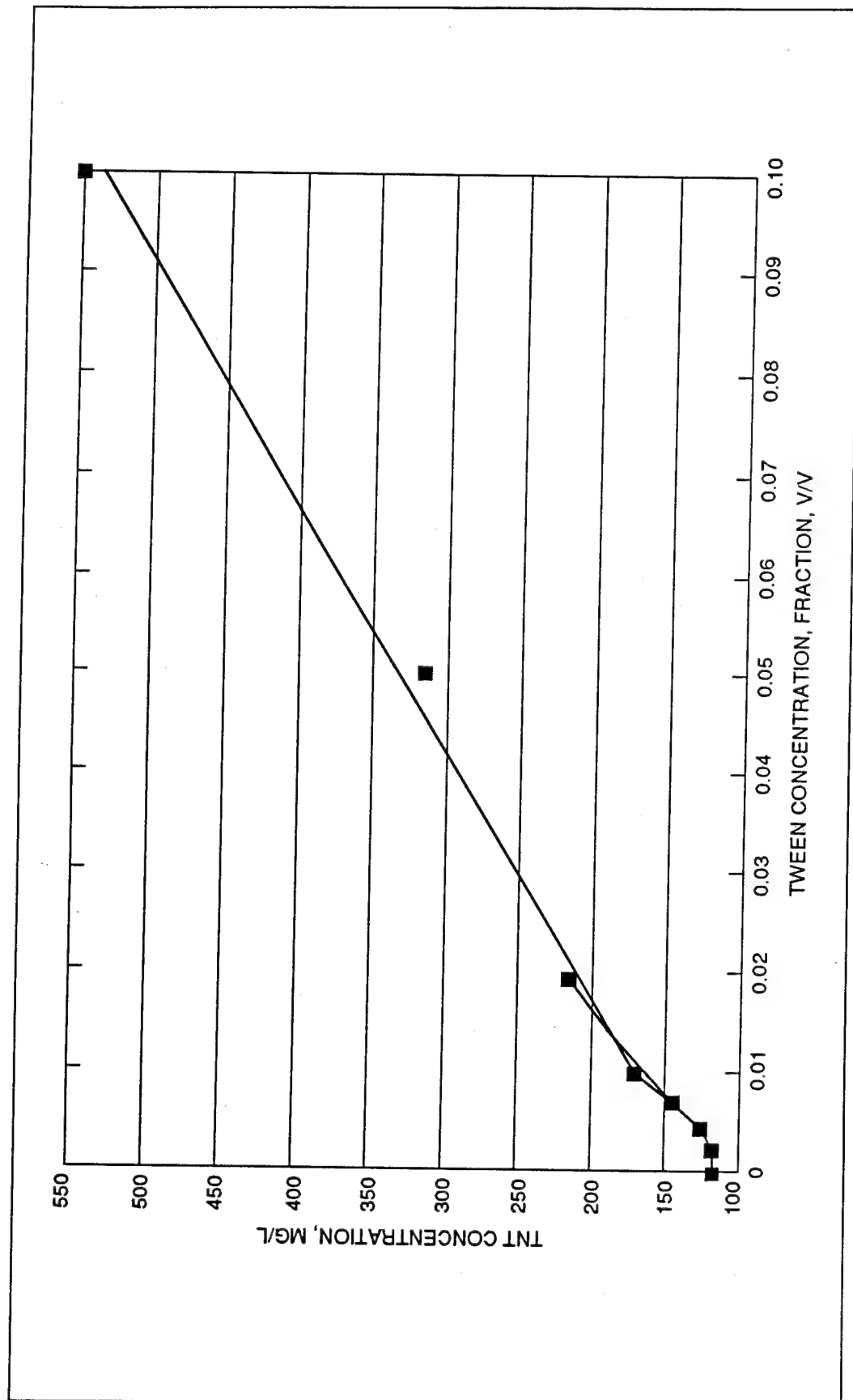


Figure 2. Relationship between Tween 80 concentration and the level of TNT in a fully saturated solution of TNT (Initial saturation level was 120 mg of TNT/L; figure is composite of two straight lines breaking at a Tween 80 concentration of 0.014 mg/L.)



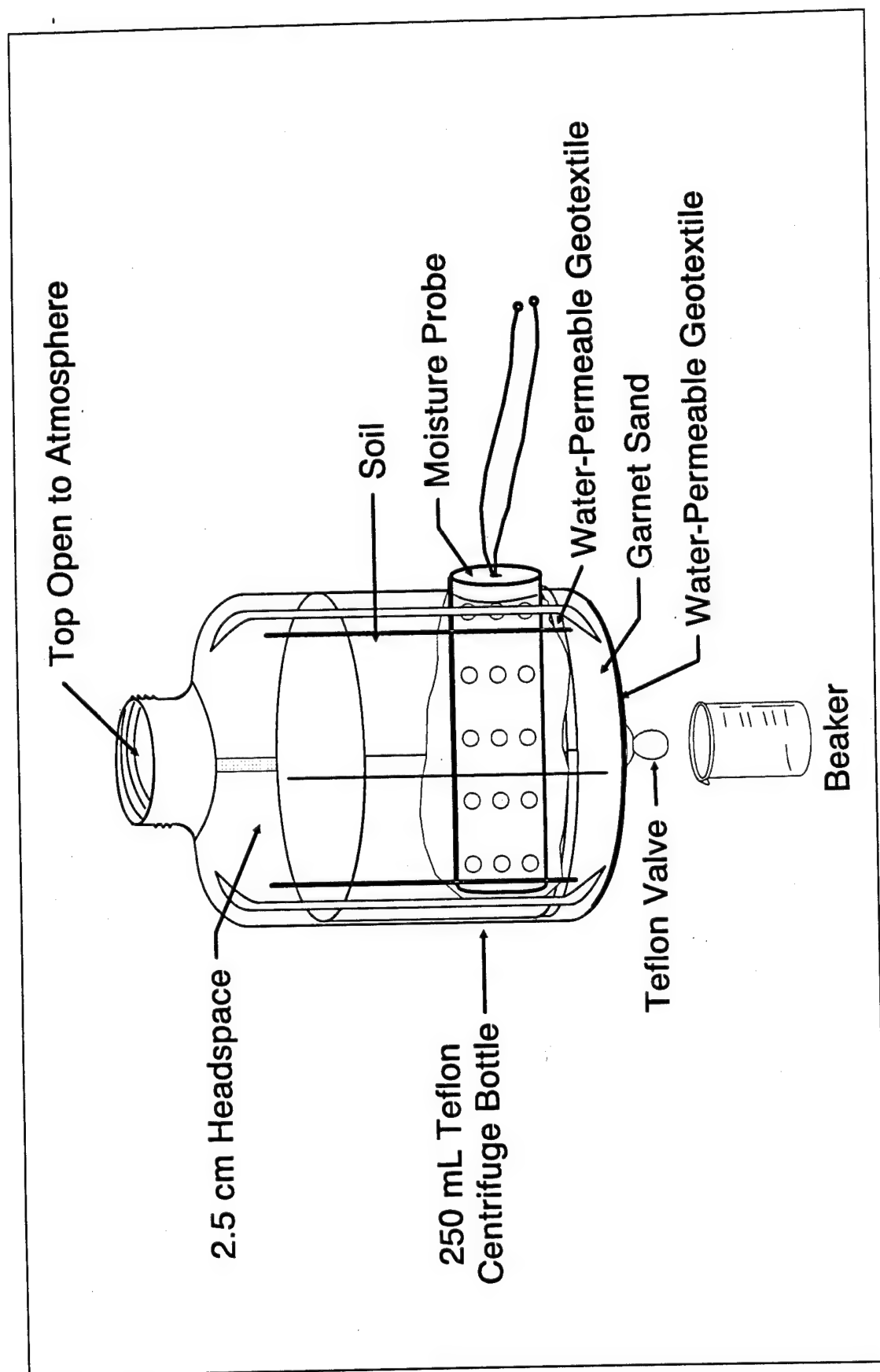


Figure 3. Basic design of the static soil treatment cell

CA) was soaked overnight in RO water, dried, and then placed in the static soil cell with teflon tape wrapped around it to form a seal. The cell was then placed into the sliding tray of the static incubation chamber. Moisture readings were determined periodically throughout the experiment with a Watermark meter (Irrrometer Company, Riverside, CA).

### **Soil incubation chamber**

Black plexiglass boxes (incubation chambers) containing removable trays, each holding forty 250-mL centrifuge tubes (cells) were used (Figure 4). Each chamber was equipped with a vented door that folded down to permit access to the trays for the loading and removal of test cells. Each tray was also equipped with two rows of metal strips at the front for use in measuring the moisture level in each test cell (Figure 4). When samples were incubated, each chamber was closed to prevent light from reacting with the TNT in the soil. A small muffin fan at the rear of the chamber gently pulled air through the unit to prevent saturation of the air with moisture and stagnation of the air in the box.

### **Development of moisture dosing procedures**

Table 1 shows that 132 g of WES reference soil DWE were loaded into each of four cells (Figure 3). One soil received no treatment. To simulate surface application, the remaining three cells were sprayed evenly with distilled water applied in quantities of 10, 20, or 30 mL, respectively, using a pipetting machine fitted with a distribution wand (Figure 5). These cells were incubated at room temperature and monitored periodically for the detection of moisture at the probe surface.

To simulate the incorporation of moisture and nutrients by mixing, three additional cells were loaded with probes and 132 g DWE of soil that had been moistened with 10, 20, and 30 mL of distilled water, respectively. These cells were placed vertically on a reciprocating shaker and shaken continuously, except for brief periods when readings were taken.

### **Loading and treatment of soil in test cells**

As described above, 132 g DWE of soil were placed into each cell. Abiotic controls were established by adding 16.7 g  $\text{HgCl}_2/\text{kg}$  DWE of soil and mixing the material before placing it into the control cells.

Active experimental treatments consisted of two blocks of twelve cells each (three replicates for each of four sample times) for the high nutrient and low nutrient treatments (Table 2). Control treatments consisted of two blocks of eight cells each (two replicates for each of four sample times) for the biotic

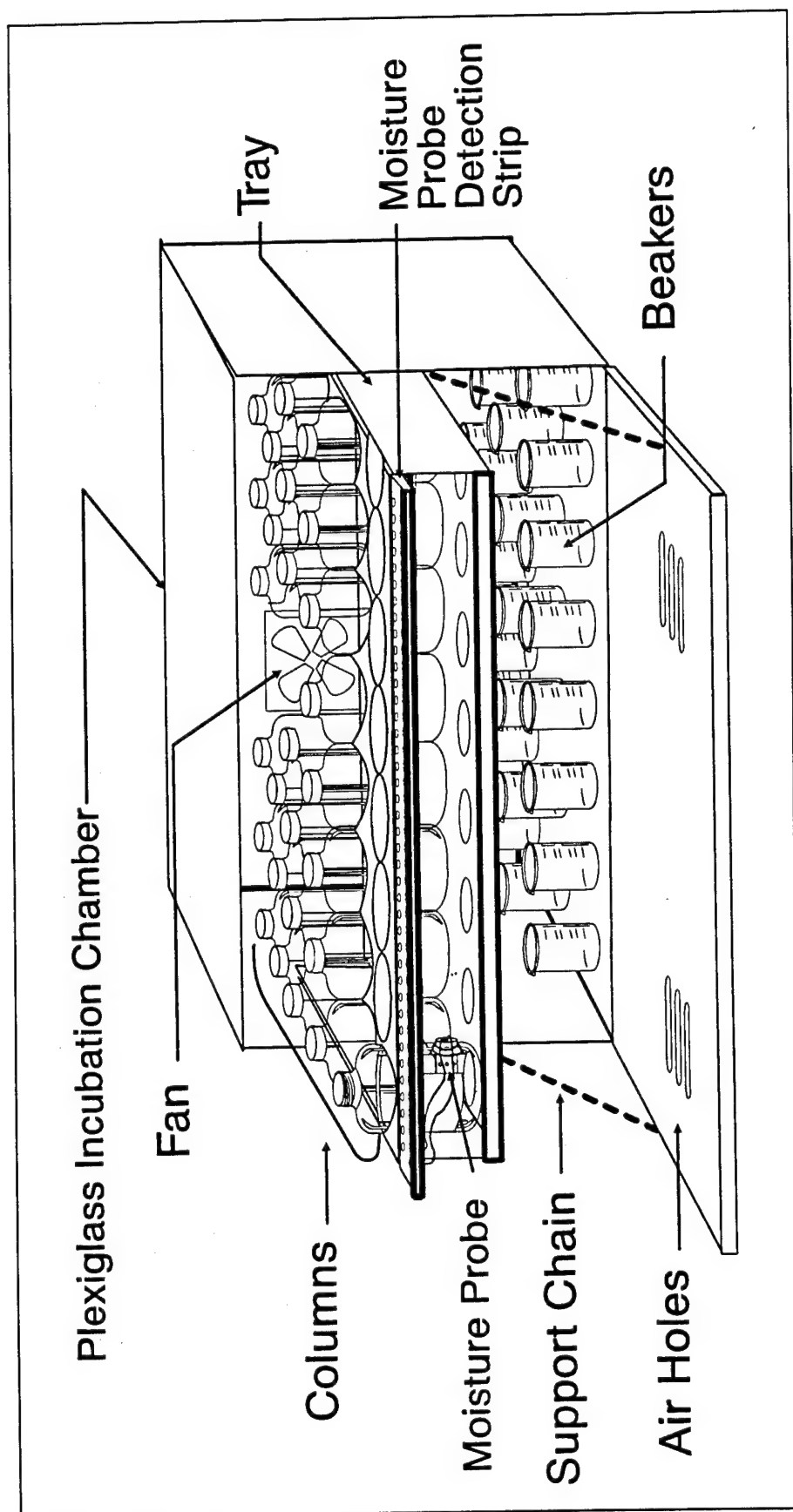


Figure 4. Configuration of the soil incubation chamber

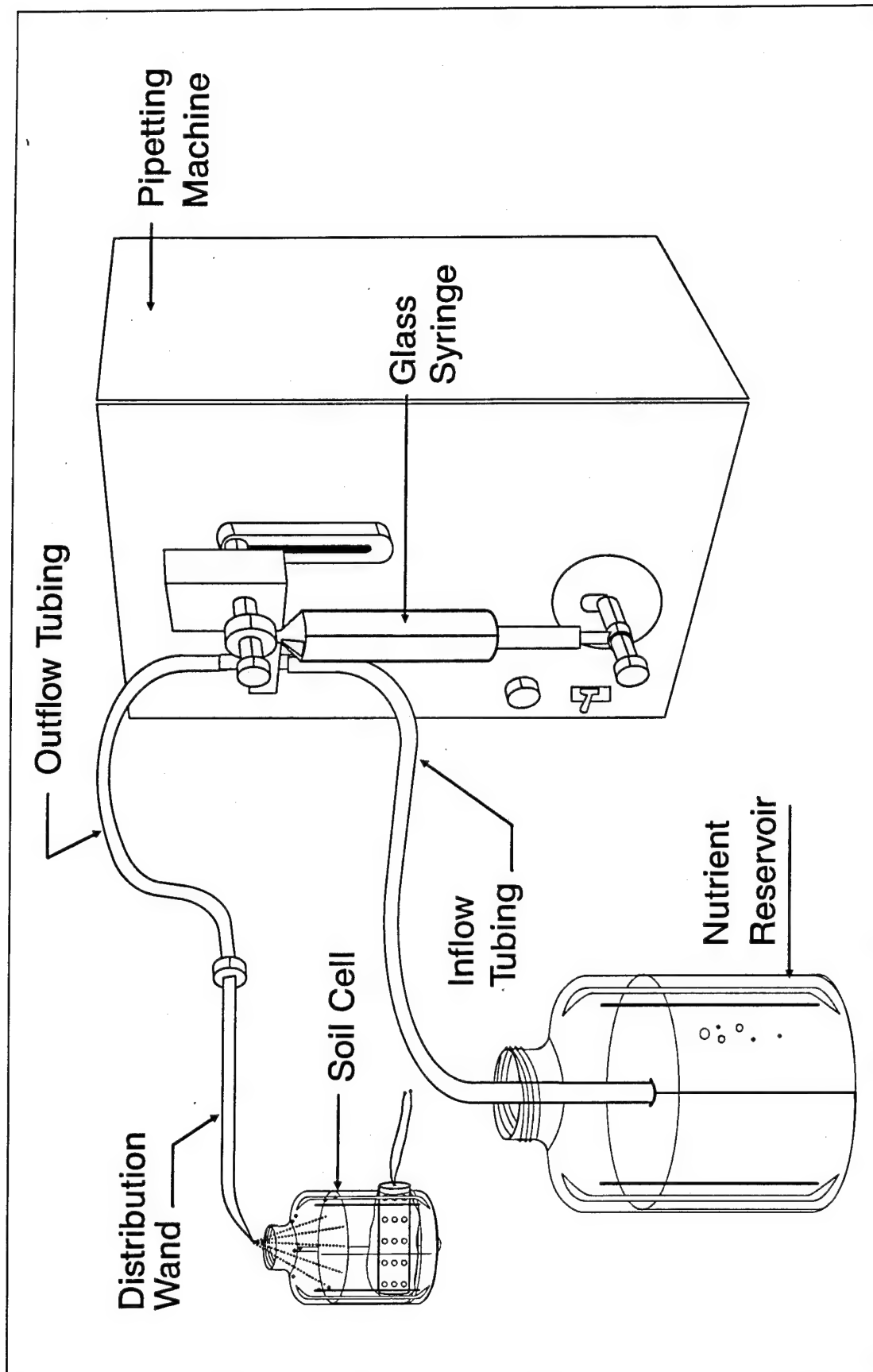


Figure 5. Automatic pipetting machine with nutrient reservoir and distribution wand

Table 2 Treatments for Static Soil Cell Study	
Treatment	Components Added to Nutrient Solution
Biotic Control	None - water only
Low Nutrient Treatment	Tween 80 - 14.0 g/L $\text{NH}_4\text{NO}_3$ - 126 mg/L $\text{K}_2\text{HPO}_4$ - 18 mg/L $\text{KH}_2\text{PO}_4$ - 18 mg/L Sodium acetate - 6.75 g/L
High Nutrient Treatment	Tween 80 - 14.0 g/L $\text{NH}_4\text{NO}_3$ - 1,260 mg/L $\text{K}_2\text{HPO}_4$ - 180 mg/L $\text{KH}_2\text{PO}_4$ - 180 mg/L Sodium acetate - 67.5 g/L
Abiotic Control	$\text{HgCl}_2$ - Mixed with soil during cell preparation @ 16.7 g/Kg Tween 80 - 14.0 g/L $\text{NH}_4\text{NO}_3$ - 1,260 mg/L $\text{K}_2\text{HPO}_4$ - 180 mg/L $\text{KH}_2\text{PO}_4$ - 180 mg/L Sodium acetate - 67.5 g/L

and abiotic controls. Nutrient treatments were each sprayed with 30 mL of the appropriate autoclaved nutrient solution. Biotic controls received 30 mL of sterile RO water, while abiotic controls received 30 mL of sterile high nutrient solution. Untreated samples were taken in triplicate prior to test cell loading to determine time 0 levels of microorganisms and soil contaminants.

Readings of the moisture probes were taken 4 times per week. When cells reached 25 to 30 centibars of resistance, the appropriate nutrient solution was reapplied to each cell. Air purging was initially conducted four times per week to determine microbial activity through the production of  $\text{CO}_2$  and to replace spent  $\text{O}_2$ . Carbon dioxide-free air was forced into the cells through the drain valve on the bottom at a rate of 1 mL/min. Air exiting the cells was passed over an oxygen meter probe (Engineered Systems and Designs, Model III, Newark, DE). When monitoring a cell, an oxygen reading was taken every minute until readings stabilized for 3 consecutive minutes. Stabilization times ranged from 8 to 30 min. Since no detectable decreases in oxygen concentrations were observed over the course of the study, air purging frequencies were changed from four times per week to once per week.

### Sample collection and processing

Static cells were sacrificed at 2.5, 4, 6, and 7 months of incubation. To monitor the actual disappearance of TNT from the most active treatment, soils were removed from the cell with a sterile spatula and subsampled for dry weight, TNT and TNT transformation products, and microbial enumeration.

## Microbial Enumeration Procedures

Two media were used to enumerate microorganisms from the shake flask and static cell tests: (a) a basal salts medium with yeast extract and 100 mg of TNT, 10 g of cosubstrate (other than toluene), and 15 g of agar per liter (BSA-TNT); and (b) a peptone-tryptone-yeast extract-glucose agar (PTYG). When toluene was used as the cosubstrate, BSA-TNT plates were incubated in closed containers in the presence of toluene fumes. The compositions of these media are presented in Tables 3 and 4.

**Table 3**  
**Composition of BSA Medium for Isolation and Growth of Bacteria Degrading Explosives<sup>1,2,3</sup>**

Component	Chemical Formulation	Amount <sup>2</sup>	10X Strength
Ammonium Sulfate, g	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.4	4.0
Potassium Phosphate, Dibasic, g	K <sub>2</sub> HPO <sub>4</sub>	0.1	1.0
Potassium Phosphate, Monobasic, g	KH <sub>2</sub> PO <sub>4</sub>	0.05	0.5
Magnesium Sulfate, Heptahydrate, g	MgSO <sub>4</sub> * 7H <sub>2</sub> O	0.05	0.5
Manganese Chloride, Tetrahydrate, g	MnCl <sub>2</sub> * 4H <sub>2</sub> O	0.02	0.2
Calcium Chloride, Dihydrate, g	CaCl <sub>2</sub> * 2H <sub>2</sub> O	0.005	0.05
Ferrous Chloride, Tetrahydrate, g	FeCl <sub>2</sub> * 4H <sub>2</sub> O	0.005	0.05
Calcium Carbonate, g	CaCO <sub>3</sub>	0.2	omit
Tap Water (as needed), L		1 L	1 L
Agar, g	N/A	15.0	omit

<sup>1</sup> Modified from Aaronson's Medium for Enrichment and Isolation of *Pseudomonas* Capable of Oxidizing Naphthalene (Aaronson 1970).

<sup>2</sup> For normal strength medium, add each of the ingredients in the order listed to at least 800 mL of tap water while stirring. Wait until the last ingredient added has dissolved before adding the next. Filter the final medium through a fine filter paper or a 0.45- $\mu$ m micropore filter before adding any organic ingredients or agar and sterilizing. For the 10X medium, again add the ingredients, except for CaCO<sub>3</sub>, to at least 800 mL of tap water. Store in refrigerator. Since no organics will be added to the 10X medium, it should be good for several weeks. When ready to use, dilute 100 mL of the 10X to 1 L with tap water, add 0.2 g of CaCO<sub>3</sub> while stirring, and filter as for normal strength medium. Then add any remaining ingredients and sterilize.

<sup>3</sup> The normal pH of this medium is 6.9 to 7.0.

**Table 4**  
**Composition of PTYG Medium for Isolation of Total Heterotrophic Microorganisms<sup>1</sup>**

Component	Chemical Formulation	Amount	10X Strength
Glucose, g	C <sub>6</sub> H <sub>12</sub> O <sub>6</sub>	0.5	5.0
Peptone, g	N/A	0.5	5.0
Tryptone, g	N/A	0.25	2.5
Yeast Extract, g	N/A	0.5	5.0
Magnesium Sulfate, g	MgSO <sub>4</sub>	0.25	2.5
Calcium Chloride, g	CaCl <sub>2</sub>	0.07	0.7
Agar, g	N/A	15.0	omit
RO Water, L		1 L	1 L

<sup>1</sup> The pH of this medium can be adjusted to the prevailing pH value of the soil. However, for this work, the pH was maintained at 6.9 to 7.0.

Numbers of microorganisms were determined by diluting 1-g DWE of soil from each treatment in sterile phosphate buffer from 10<sup>-1</sup> to 10<sup>-9</sup> and plating each onto a PTYG and BSA-TNT medium. Numbers of microorganisms were determined after 1 to 3 weeks of incubation at room temperature. Bacteria recovered on a PTYG medium were considered total heterotrophic bacteria. Bacteria recovered on a BSA-TNT medium plus a cosubstrate were considered able to tolerate or utilize TNT. However, this medium does not distinguish between those microorganisms growing on TNT and those growing on the cosubstrate alone. Generally, however, some cosubstrate is required to support the growth of TNT-degrading consortia (Gunnison et al. 1993).

## Chemical Analysis

Soils were analyzed for particle size composition using Patrick's (1958) method. Total organic carbon content in soil samples was determined by dry combustion (Allison 1965). Soil moisture content was determined as indicated previously.

The TNT, and the 2ADNT, 4ADNT, 2,4DA6NT, and 2,6DA4NT transformation products were measured by EPA SWA 846, Number 8330 (EPA 1990). This method requires extraction with acetonitrile and analysis by high pressure liquid chromatography (HPLC). Analyses were performed on a Hewlett Packard HPLC having a 600 MS System Controller/Solvent Delivery System, a 700 Satellite WIST Injector, and a 991 MS Photodiode Array Detector. Separation was accomplished using a Supelco LC-18 25-cm by 4.6-mm cell having a pore size of 5 µm eluted with 30 percent acetonitrile in

water at a flow rate of 1.2 mL/min. The TNT and the 2ADNT, 4ADNT, 2,4DNT, 2,6DNT, 1,3,5-trinitrobenzene (TNB), 2,4,6-triaminotoluene (TAT) and 1,3-dinitrobenzene (DNB) analytes were confirmed using analytical reference standards.<sup>1</sup> An analysis for azoxytoluene compounds in static cell soil samples was conducted by extraction with 100 percent acetonitrile. Separation of azoxytoluenes on the HPLC was accomplished with a gradient ranging from 30 percent acetonitrile in water to 100 percent acetonitrile. The compounds 4,4',6,6'-tetranitro-2, 2'-azoxytoluene and 2,2',6,6'-tetranitro-4,4'-azoxytoluene were used as standards for the azoxy compounds.<sup>1</sup>

## Statistical Analysis

Statistical analysis of the data was conducted with the SigmaStat<sup>TM</sup> statistical software system (Jandel Scientific, San Rafael, CA) using one-way analysis of variance followed by the Student-Newman-Keuls method for pairwise multiple comparisons. Graphs and bar charts were constructed using the SigmaPlot<sup>TM</sup> graphing system (Jandel Scientific, San Rafael, CA).

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<sup>1</sup> Standards provided by Dr. Ronald Spangord, SRI Inc., Menlo Park, CA.



## 3 Results and Discussion

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### Shake Test Results—Enumeration of Microorganisms

#### Bangor soil

The number of total heterotrophic bacteria initially present in Bangor soil was approximately  $10^5$  colony-forming units (CFUs)/g DWE (Figure 6). After 30 days of incubation, the numbers of bacteria recovered from the biotic control had increased to approximately  $10^7$  CFUs/g. No microorganisms were recovered from the acidified control. Nutrient levels alone had little impact on the numbers of heterotrophs compared to the biotic control. However, nutrients in combination with toluene, Tween 80, or with both increased the numbers significantly. These results demonstrate that toluene can serve as a cosubstrate to support microflora in this soil. Furthermore, nutrients plus Tween 80 or toluene alone or together will be sufficient to support an active heterotrophic population. In addition to enhancing desorption of TNT from soil, Tween 80 may also serve as a cosubstrate to support the growth of microflora. Since microorganisms were not recovered on the BSA-TNT media (results not shown), TNT degraders were either not present in this soil, or required nutrient(s) were not provided by the BSA-TNT medium.

#### Crane Sifter Conveyor soil

Total heterotrophic bacteria (PTYG agar) present at the start of incubation in the Crane Sifter Conveyor soil were also approximately  $10^5$  CFUs/g DWE of soil (Figure 7). After 30 days of incubation, numbers of microorganisms in the acidified controls fell to  $10^4 \pm 751$  CFUs/g DWE of soil, suggesting that the HCl was successful in killing most, but not all, of these microorganisms. Most of the low nutrient treatments and the high nutrient plus Tween 80 treatment exceeded the biotic control ( $10^9$  CFUs/g DWE), while the remaining treatments were more or less equal to the biotic control ( $10^7$  to  $10^9$  CFUs/g DWE). The high nutrient dose alone, with toluene, or with Tween 80 and toluene in combination was unable to support growth in excess of the biotic control. Moreover, the low nutrient dose plus Tween 80 and toluene inhibited growth.

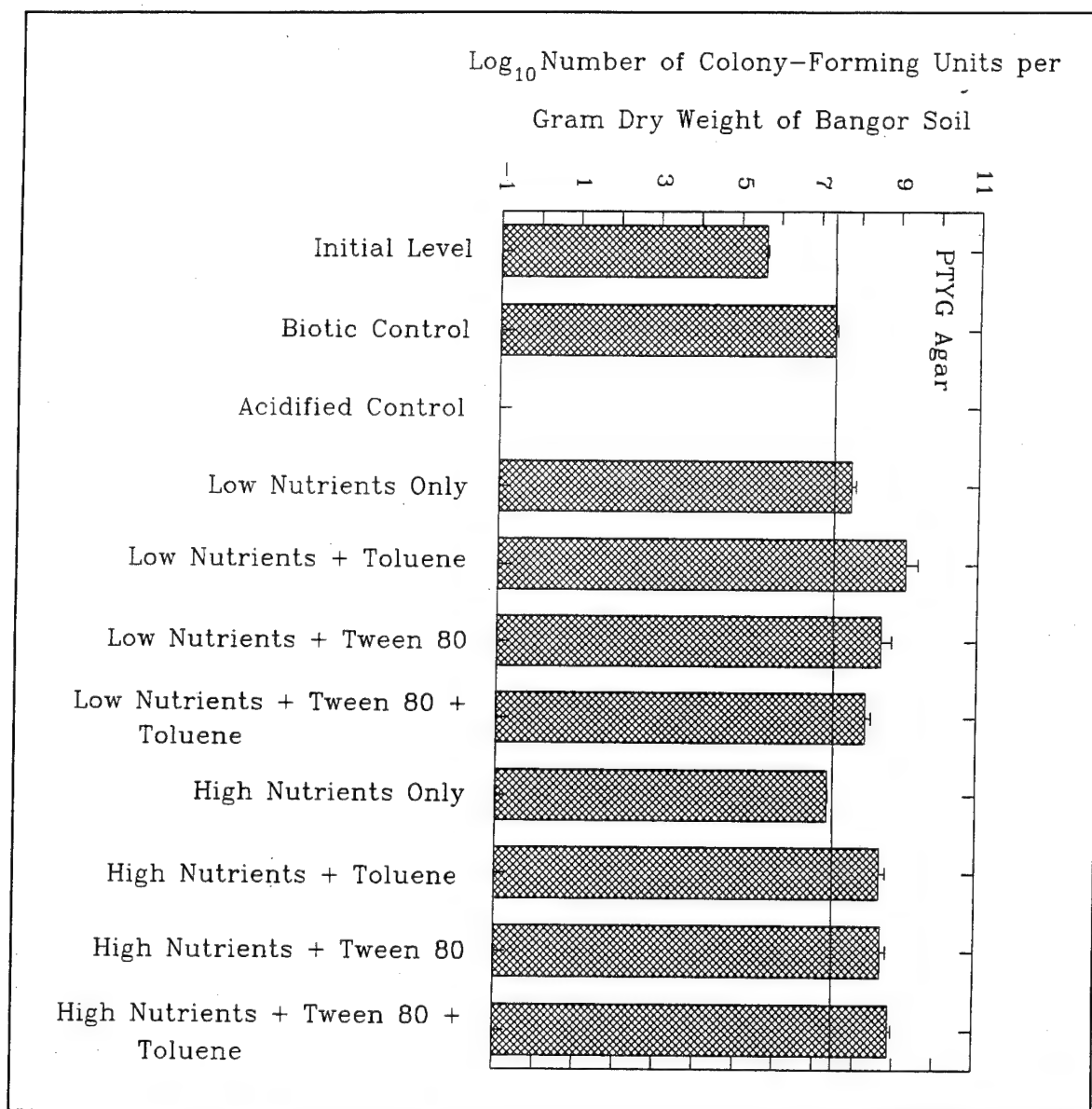


Figure 6. Response of native heterotrophic bacteria cultured on PTYG agar to treatment with various combinations of additives in the shake flask test with Bangor soil (No microorganisms were recovered on BSA-TNT medium. Bars are the means of 3 replicates  $\pm$  standard error of the mean. The horizontal line marks total heterotrophs in the biotic control.)

When assayed on BSA-TNT medium, microorganisms from Crane Sifter soils grew on the biotic and acidified controls and on the low nutrients plus toluene both with and without Tween 80 (Figure 7). However, growth on the latter media was virtually the same as the acidified control and was exceeded by that recovered on the biotic control. Apparently, a steady source of moisture was sufficient to support the activity of microorganisms recovered on this medium. Additional substrates were somewhat inhibitory. This particular soil also contains a high concentration of 1,3,5-hexahydro-1,3,5-trinitrotriazine

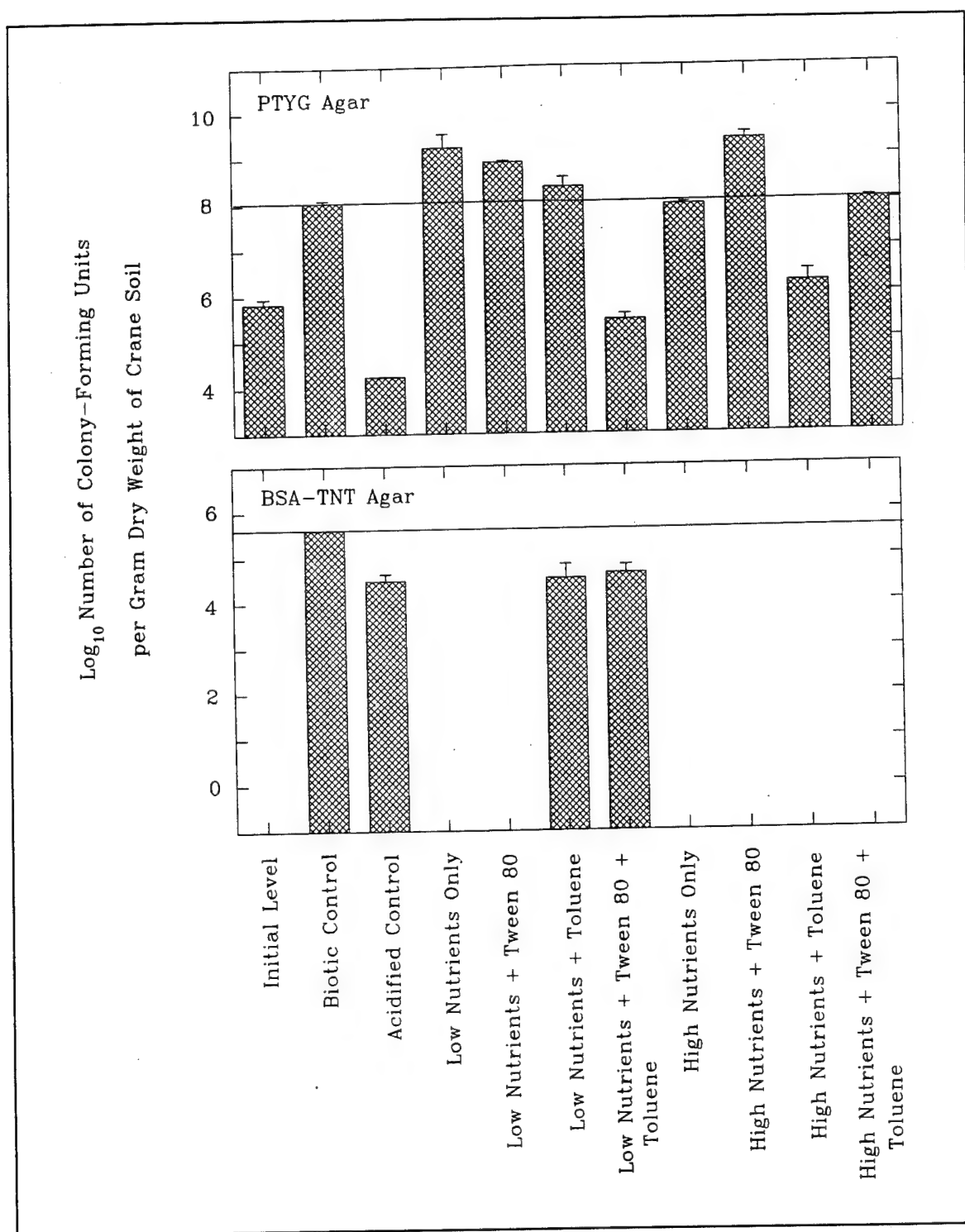


Figure 7. Response of native heterotrophic bacteria cultured on PTYG agar and bacteria cultured on BSA-TNT medium to treatment with various combinations of additives in the shake flask test with Crane soil (Bars are the means of 3 replicates  $\pm$  standard error of the mean. The horizontal line marks total heterotrophs in the biotic control.)

(RDX) and 1,3,5,7-tetranitro-1,3,5,7-octahydrotetrazocine (HMX) levels as high as TNT (see sorption report by Pennington et al. in press). This mixture of explosives in combination with high nutrients may have been toxic, particularly when Tween 80 and toluene were also included.

### **Hastings soil**

Initial levels of total heterotrophic bacteria (PTYG agar) in the Hastings soil were also approximately  $10^5$  CFUs/g DWE (Figure 8). The acidified controls had no bacteria at 30 days, indicating that poisoning with acid was successful in preventing growth. The levels of microorganisms from most of the remaining treatments equalled or exceeded the biotic controls, often reaching  $10^8$  CFUs/g DWE in 30 days.

When the Hastings soil samples were plated onto BSA-TNT plus a cosubstrate (biotic control), the colony count rose from below detection in the initial samples to  $10^7$  CFUs/g DWE (Figure 8). The results demonstrate that nutrients with and without Tween 80 will enhance (increase) numbers of TNT-degrading microorganisms in Hastings soil.

### **McAlester soil**

Initial levels of heterotrophic bacteria (PTYG agar) in McAlester soil were  $10^7$  CFUs/g dry weight of soil (Figure 9). Not all of the heterotrophic bacteria were killed in the acidified control, and low levels of these microorganisms remained at 30 days. Nutrients increased the numbers of total heterotrophic bacteria, except when toluene was added. However, toluene exerted no effect if Tween 80 were added. These results demonstrated that toluene cannot serve as a cometabolite for treatment of this soil. Nutrients and Tween 80 alone are sufficient.

When the microorganisms from these treatments were plated on BSA-TNT agar, the number of microorganisms recovered from the biotic control decreased approximately two orders of magnitude (Figure 9). Microorganisms recovered from the active treatments without toluene remained at the same level as on PTYG agar, suggesting that the same microorganisms may have been recovered on both media. By contrast, the number of microorganisms recovered from the low nutrients plus toluene treatment increased by approximately one order of magnitude on the BSA-TNT agar, indicating that the low nutrient plus toluene treatment supported more microorganisms able to utilize toluene as a cosubstrate than on the PTYG medium. No microorganisms were recovered on BSA-TNT agar inoculated with samples from the other toluene-containing treatments. Based on microbial recoveries obtained on both media, the treatments of choice for this soil are the low nutrients with Tween 80 and the high nutrients plus Tween 80, followed closely by the low nutrients only and the low nutrients plus toluene treatments.

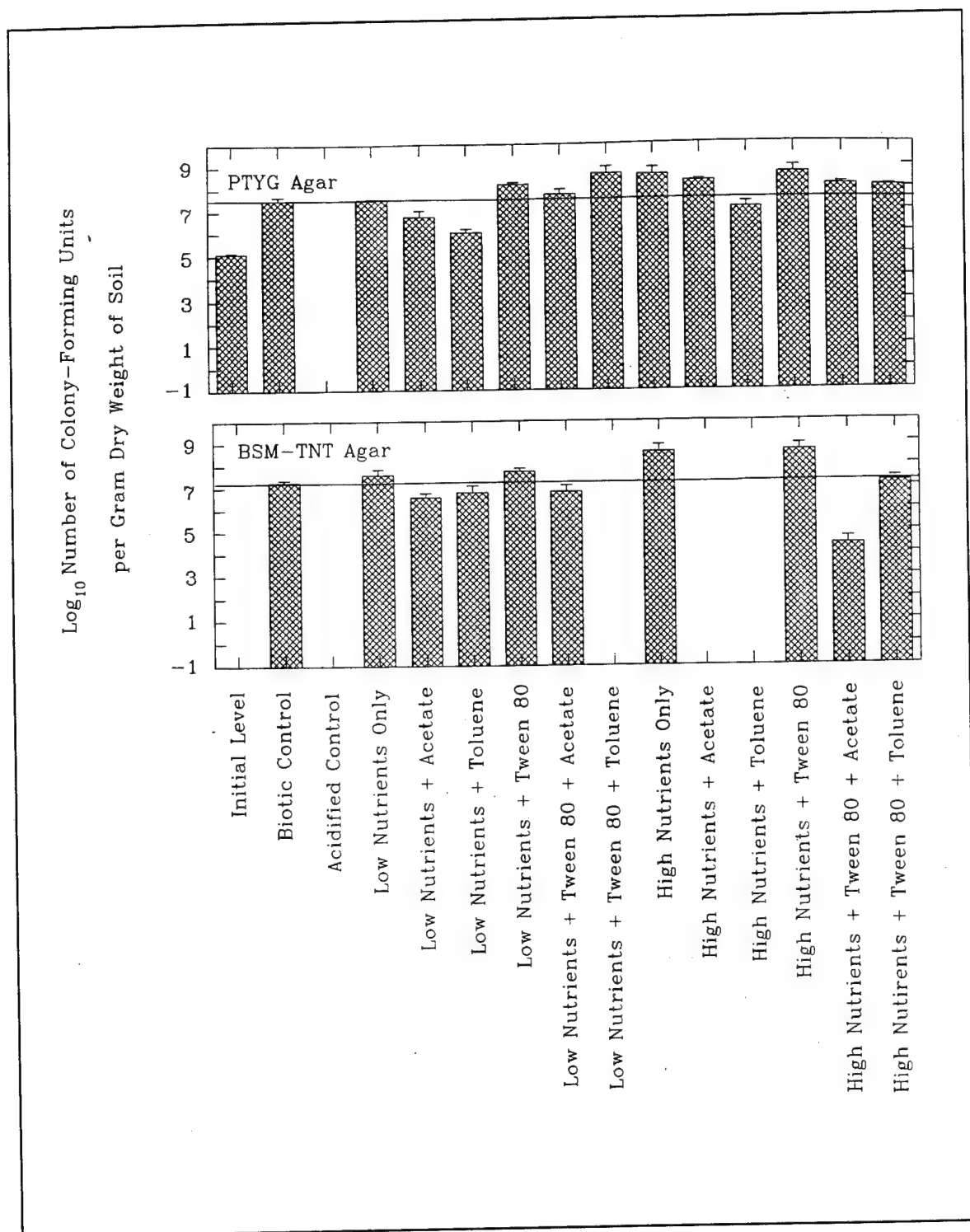


Figure 8. Response of native heterotrophic bacteria cultured on PTYG agar and bacteria cultured on BSA-TNT medium to treatment with various combinations of additives in the shake flask test with Hastings soil (Bars are the means of 3 replicates  $\pm$  standard error of the mean. The horizontal line marks total heterotrophs in the biotic control.)

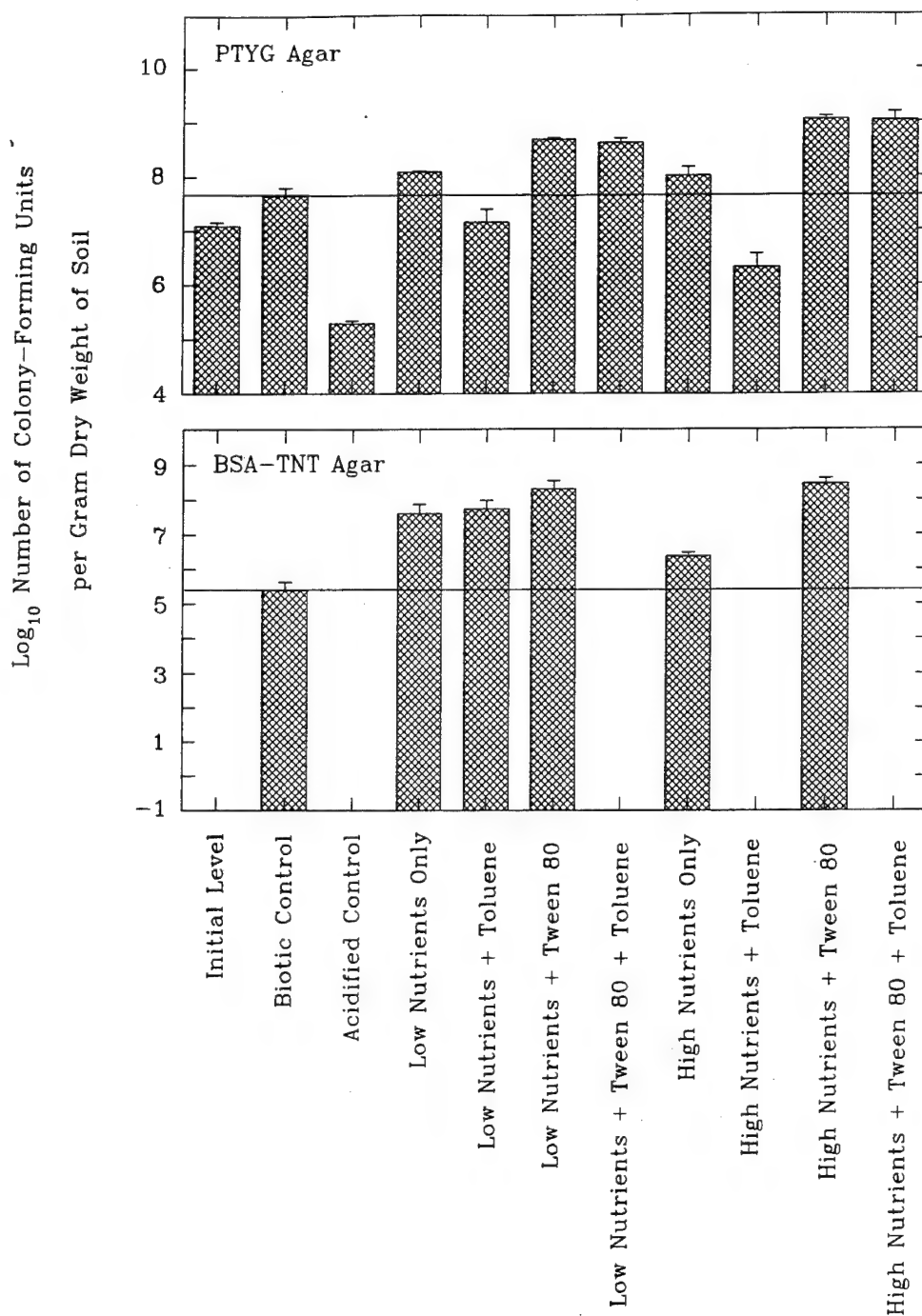


Figure 9. Response of native heterotrophic bacteria cultured on PTYG agar and bacteria cultured on BSA-TNT medium to treatment with various combinations of additives in the shake flask test with McAlester soil (Bars are the means of 3 replicates  $\pm$  standard error of the mean. The horizontal line marks total heterotrophs in the biotic control.)

## "Fate" of TNT in the soils tested

Detailed descriptions of the fate of TNT and TNT transformation products for each of the soils based on the testing are presented in Tables 5 through 8, while the overall results for TNT are summarized in Figure 10(a). Low nutrients, low nutrients plus toluene, and high nutrients plus toluene were generally most effective in supporting some disappearance of TNT. Generally, the persistence of TNT increased as the initial levels of this compound in soil increased.

Based on our present understanding, at least part of the fate of TNT under aerobic conditions is related to the information of transformation products, such as 2ADNT, 4ADNT, and TNB, as well as some other compounds not found in this study. Increases in the levels of 2ADNT were supported by the low nutrient and sometimes the high nutrient plus toluene treatments (Tables 5 through 8 and Figure 10(b)). All treatments, including the acidified control, increased 4ADNT in Hastings soil, while the low nutrient only treatment increased the level of 4A in Crane sifter soil (Figure 10(c)). In a similar manner, most treatments increased TNB in each of the soils except Bangor; high nutrients caused an especially pronounced increase in McAlester soil (Figure 10(d)).

## Variability of data

The TNT levels in the 30-day samples were extremely variable. The variability may be related to abiotic and biotic transformational processes active on TNT. With McAlester soil data, variations in levels of each constituent at time 0 were quite similar and quite small (around 1 percent of the mean value). However, the final values indicate that while variations for TNT levels increased markedly during treatment, those for RDX and HMX were similar to the initial variations as illustrated in the following tabulation:

Constituent	Initial Value	Final Value
TNT	3,390 $\pm$ 75.1	3,160 $\pm$ 1,940
RDX	808 $\pm$ 9.74	94 $\pm$ 1.03
HMX	213 $\pm$ 2.09	16 $\pm$ 0.204

Similar results were observed for the other soils. This suggests that some interaction of TNT with treatment matrices occurs. Possible mechanisms of interactions include adsorption, chemical transformation (abiotic transformation and/or polymer formation), and biological (susceptibility to biotransformation and/or complete mineralization) properties. For a discussion of the role of abiotic transformation processes, see Pennington et al. (in press). These mechanisms would be more dependent upon uncontrolled variables in

**Table 5**  
**Changes in Levels of TNT, 2ADNT, and 4ADNT in Response to Nutrient, Surfactant, and Cosubstrate Amendments to Bangor Soil**

Treatment	Level of TNT and TNT By-Products, mg/kg Dry Weight <sup>1,2</sup>				
	TNT	2ADNT	4ADNT	DNT <sup>3</sup>	TNB
Initial	2.10 ± 0 <sup>b</sup>	0.615 ± 0.011 <sup>c</sup>	1.43 ± 0.222 <sup>c</sup>	<0.250	<0.250
Biotic control	3.81 ± 0.351 <sup>a</sup>	0.462 ± 0.024 <sup>d</sup>	1.96 ± 0.085 <sup>c</sup>	<0.250	<0.250
Abiotic control	2.02 ± 1.01 <sup>b</sup>	0.500 ± 0.029 <sup>c,d</sup>	2.01 ± 0 <sup>c</sup>	<0.250	<0.250
Low nutrient	<0.250 <sup>c</sup>	0.531 ± 0.022 <sup>d</sup>	<0.250 <sup>d</sup>	<0.250	<0.250
Low nutrient plus toluene	<0.250 <sup>c</sup>	0.319 ± 0.015 <sup>e</sup>	1.62 ± 0.362 <sup>c</sup>	<0.250	<0.250
Low nutrient plus Tween	<0.250 <sup>c</sup>	0.745 ± 0 <sup>a</sup>	2.87 ± 0.195 <sup>b</sup>	<0.250	<0.250
Low nutrient plus Tween 80 plus toluene	<0.250 <sup>c</sup>	0.644 ± 0.022 <sup>b</sup>	7.57 ± 6.19 <sup>a</sup>	<0.250	<0.250
High nutrient	<0.250 <sup>c</sup>	0.613 ± 0.023 <sup>c</sup>	1.43 ± 0.043 <sup>c</sup>	<0.250	<0.250
High nutrient plus toluene	<0.250 <sup>c</sup>	0.330 ± 0 <sup>e</sup>	2.98 ± 0.298 <sup>b</sup>	<0.250	<0.250
High nutrient plus Tween 80 plus toluene	<0.250 <sup>c</sup>	<0.250 <sup>f</sup>	<0.250 <sup>d</sup>	<0.250	<0.250

<sup>1</sup> Values followed by the same letter within a given column do not differ significantly at the 95 percent confidence level. Values given are the means of two to three replicates ± standard error of the mean.

<sup>2</sup> Detection limits for TNT, 2ADNT, and 4ADNT were each 0.25 mg/kg DWE soil.

<sup>3</sup> All values in this column were below the detection limit.



**Table 6**  
**Changes in Levels of TNT, 2ADNT, and 4ADNT in Response to Nutrient, Surfactant, and Cosubstrate Amendments to Crane Sifter Conveyor Soil**

Treatment	Level of TNT and TNT By-Products, mg/kg Dry Weight <sup>1,2</sup>				
	TNT	2ADNT	4ADNT	DNT <sup>3</sup>	TNB
Initial	1,660 ± 15.3 <sup>a</sup>	33.6 ± 0.610 <sup>c</sup>	18.2 ± 1.03 <sup>c</sup>	<0.250	6.3 ± 0.468 <sup>c</sup>
Biotic control	1,300 ± 54.3 <sup>b</sup>	35.9 ± 1.11 <sup>c</sup>	21.8 ± 0.805 <sup>b</sup>	<0.250	14.0 ± 0.428 <sup>c</sup>
Abiotic control	1,760 ± 94.1 <sup>a</sup>	22.6 ± 0.554 <sup>d</sup>	20.9 ± 0.821 <sup>b</sup>	<0.250	12.9 ± 3.20 <sup>c</sup>
Low nutrient	1,710 ± 212 <sup>a</sup>	51.0 ± 1.00 <sup>b</sup>	29.0 ± 1.00 <sup>a</sup>	<0.250	14.0 ± 1.00 <sup>c</sup>
Low nutrient plus toluene	142 ± 10.9 <sup>c</sup>	47.9 ± 5.53 <sup>b</sup>	24.6 ± 0.854 <sup>a</sup>	<0.250	7.52 ± 0.752 <sup>c</sup>
Low nutrient plus Tween 80 plus toluene	1,420 ± 43.7 <sup>b</sup>	40.0 ± 2.96 <sup>b</sup>	21.7 ± 0.821 <sup>b</sup>	<0.250	33.1 ± 2.1 <sup>a</sup>
High nutrient	1,500 ± 194 <sup>a,b</sup>	42.0 ± 7.00 <sup>b</sup>	22.0 ± 1.00 <sup>b</sup>	<0.250	15.0 ± 1.00 <sup>c</sup>
High nutrient plus toluene	106 ± 27.6 <sup>c</sup>	66.3 ± 1.22 <sup>a</sup>	23.1 ± 2.36 <sup>a,b</sup>	<0.250	7.22 ± 1.36 <sup>c</sup>
High nutrient plus Tween 80 plus toluene	1,200 ± 112 <sup>b</sup>	37.6 ± 0.456 <sup>c</sup>	20.1 ± 0.86 <sup>b</sup>	<0.250	27.0 ± 1.83 <sup>b</sup>

<sup>1</sup> Values followed by the same letter within a given column do not differ significantly at the 95 percent level of confidence. Values given are the means of two to three replicates ± standard error of the mean.

<sup>2</sup> Detection limits for TNT, 2ADNT, 4ADNT, DNT, and nitrobenzenes were each 0.250 mg/kg DWE soil.

<sup>3</sup> All values in this column were below the detection limit.

Table 7

Changes in Levels of TNT, 2ADNT, and 4ADNT in Response to Nutrient, Surfactant, and Cosubstrate Amendments to Hastings Soil

Treatment	Level of TNT and TNT By-Products, mg/kg Dry Weight <sup>1,2</sup>				
	TNT	2ADNT	4ADNT	DNT <sup>3</sup>	TNB
Initial	12,800 ± 83.0 <sup>b</sup>	43.5 ± 3.01 <sup>d</sup>	<0.250 <sup>e</sup>	<0.250	73.0 ± 7.07 <sup>d</sup>
Biotic control	13,700 ± 411 <sup>b</sup>	38.3 ± 0 <sup>d</sup>	12.0 ± 0 <sup>b</sup>	<0.250	73.0 ± 7.07 <sup>d</sup>
Abiotic control	10,900 ± 167 <sup>b</sup>	52.3 ± 1.01 <sup>c</sup>	23.3 ± 0 <sup>a</sup>	<0.250	138 ± 9.32 <sup>b</sup>
Low nutrient	29,000 ± 83.1 <sup>a</sup>	63.1 ± 2.00 <sup>b</sup>	10.1 ± 1.13 <sup>c</sup>	<0.250	171 ± 6.61 <sup>a</sup>
Low nutrient plus acetate	12,300 ± 95.8 <sup>b</sup>	41.0 ± 4.36 <sup>d</sup>	13.2 ± 0 <sup>b</sup>	<0.250	230 ± 67.9 <sup>a</sup>
Low nutrient plus toluene	12,330 ± 333 <sup>b</sup>	34.3 ± 0 <sup>e</sup>	9.00 ± 1.03 <sup>c</sup>	8.96 ± 0.613 <sup>a</sup>	109 ± 7.05 <sup>c</sup>
Low nutrient plus Tween 80	24,700 ± 1,940 <sup>a</sup>	70.0 ± 5.21 <sup>a</sup>	9.11 ± 0 <sup>c</sup>	<0.250	102 ± 3.76 <sup>c</sup>
Low nutrient plus Tween 80 plus acetate	8,840 ± 1,700 <sup>c</sup>	40.6 ± 6.11 <sup>d</sup>	12.9 ± 0 <sup>b</sup>	<0.250	96.5 ± 5.81 <sup>c</sup>
Low nutrient plus Tween 80 plus toluene	13,360 ± 292 <sup>b</sup>	51.7 ± 1.11 <sup>c</sup>	14.0 ± 2.08 <sup>b</sup>	<0.250	76.4 ± 3.88 <sup>d</sup>
High nutrient	11,911 ± 1,750 <sup>b</sup>	30.1 ± 3.45 <sup>f</sup>	8.01 ± 0 <sup>d</sup>	<0.250	103 ± 16.9 <sup>c</sup>
High nutrient plus toluene	12,400 ± 708 <sup>b</sup>	36.5 ± 0 <sup>e</sup>	10.0 ± 0 <sup>c</sup>	<0.250	103 ± 0.289 <sup>c</sup>
High nutrient plus acetate	12,500 ± 250 <sup>b</sup>	36.0 ± 1.01 <sup>e</sup>	14.7 ± 0 <sup>b</sup>	<0.250	5.87 ± 0.560 <sup>e</sup>

<sup>1</sup> Values followed by the same letter within a given column do not differ significantly at the 95 percent level of confidence. Values given are the means of two to three replicates ± standard error of the means.

<sup>2</sup> Detection limits for TNT, 2ADNT, and 4ADNT were each 0.250 mg/kg DWE soil.

<sup>3</sup> Except for the value denoted "a" in this column, all values were below detection.

**Table 8**  
Changes in Levels of TNT, 2ADNT, and 4ADNT in Response to Nutrient, Surfactant, and Cosubstrate Amendments to McAlester Soil

Treatment	Level of TNT and TNT By-Products, mg/kg Dry Weight <sup>1,2</sup>				
	TNT	2ADNT	4ADNT	DNT <sup>3</sup>	TNB
Initial	3,260 ± 721 <sup>a</sup>	11.9 ± 1.22 <sup>a,b</sup>	5.10 ± 0.511 <sup>a</sup>	<0.250	1.56 ± 0.312 <sup>c</sup>
Abiotic control	2,200 ± 87.5 <sup>b</sup>	10.7 ± 4.22 <sup>b</sup>	5.51 ± 0 <sup>a</sup>	<0.250	2.25 ± 0.948 <sup>c</sup>
Biotic control	2,330 ± 3,160 <sup>b</sup>	9.00 ± 0 <sup>b</sup>	5.74 ± 0.606 <sup>a</sup>	0.970 ± 0.073 <sup>a</sup>	3.33 ± 0.290 <sup>d</sup>
Low nutrient	1,940 ± 58.3 <sup>b</sup>	9.80 ± 0.110 <sup>b</sup>	5.07 ± 0.106 <sup>a</sup>	<0.250	3.96 ± 0.191 <sup>c</sup>
Low nutrient plus toluene	2,330 ± 76.0 <sup>b</sup>	12.5 ± 0.531 <sup>a</sup>	5.43 ± 0.513 <sup>a</sup>	<0.250	4.09 ± 0.512 <sup>c</sup>
Low nutrient plus Tween 80	2,510 ± 12.5 <sup>b</sup>	7.31 ± 0.635 <sup>c</sup>	5.80 ± 0.337 <sup>a</sup>	<0.250	36.2 ± 1.04 <sup>a</sup>
Low nutrient plus Tween 80 plus toluene	2,140 ± 83.3 <sup>b</sup>	9.94 ± 0.303 <sup>b</sup>	4.16 ± 0.241 <sup>b</sup>	<0.250	27.8 ± 2.28 <sup>b</sup>
High nutrient	2,280 ± 12.5 <sup>b</sup>	7.73 ± 0 <sup>c</sup>	5.74 ± 0.117 <sup>a</sup>	0.370 ± 0.016 <sup>b</sup>	29.5 ± 0.267 <sup>b</sup>
High nutrient plus toluene	1,220 ± 0 <sup>c</sup>	10.4 ± 0.711 <sup>b</sup>	4.10 ± 0.332 <sup>b</sup>	<0.250	3.48 ± 0.861 <sup>c</sup>
High nutrient plus Tween 80	2,170 ± 267 <sup>b</sup>	13.6 ± 2.45 <sup>a</sup>	5.64 ± 0.822 <sup>a</sup>	<0.250	3.60 ± 0.131 <sup>a</sup>
High nutrient plus Tween 80 plus toluene	2,450 ± 950 <sup>a</sup>	10.3 ± 1.38 <sup>b</sup>	3.95 ± 0.409 <sup>c</sup>	0.302 ± 0.001 <sup>c</sup>	4.71 ± 0.690 <sup>c</sup>

<sup>1</sup> Values followed by the same letter within a given column do not differ significantly at the 95 percent level of confidence. Values given are the means of two to three replicates ± standard error of the mean.

<sup>2</sup> Detection limits for TNT, 2ADNT, and 4ADNT were each 0.250 mg/kg DWE soil.

<sup>3</sup> Except for the values denoted "a," "b," and "c" in this column, all values were below detection.

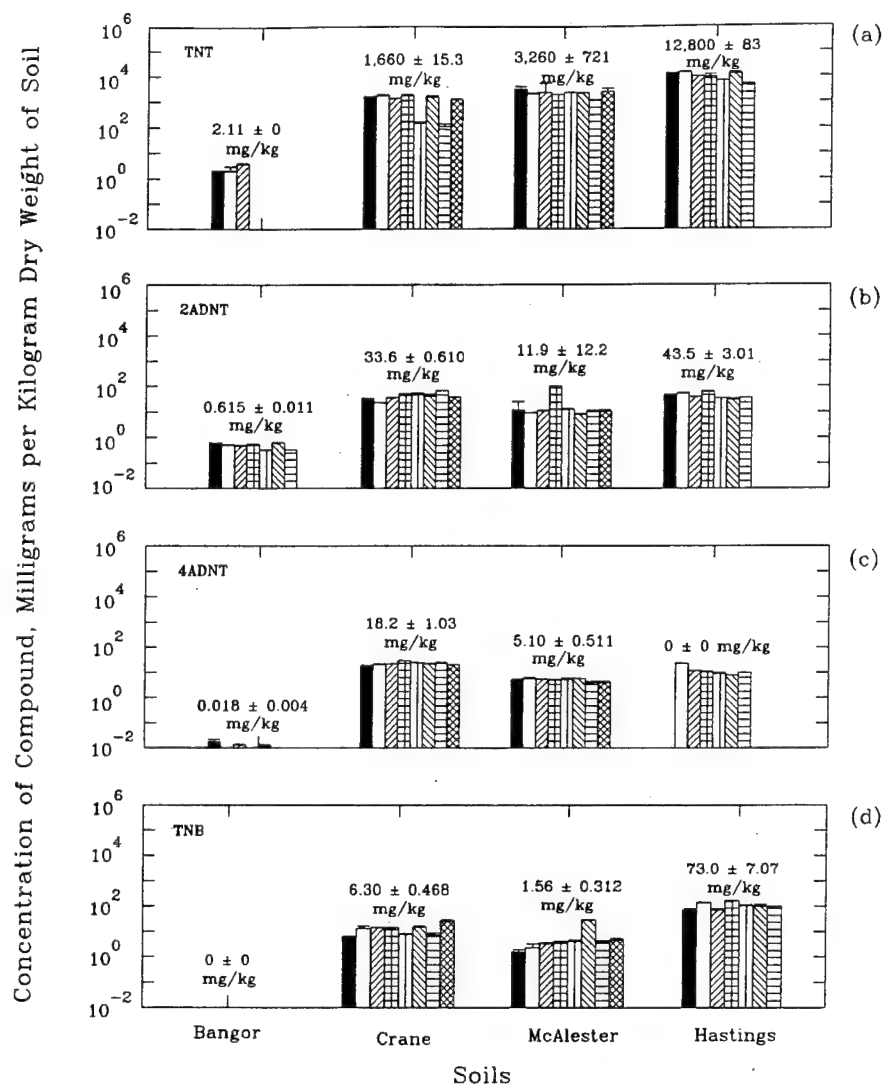


Figure 10. Comparison of removal of TNT and accumulation or removal of the TNT transformation products 2ADNT and 4ADNT and TNB in the shake test (Values given above each set of bars and the first bars in each set are the levels of each compound present at the start of treatment. The remaining bars in each set are the levels present at the end of 30 days of treatment, as indicated in the figure legend. Values given are the means of three replicates  $\pm$  the standard error of the mean.)

the experiments. These variables may include total organic carbon and the nature of the organic carbon, cation exchange capacity, and pH.

Since current understanding of these mechanisms is limited, control of them is difficult and beyond the scope of this experiment. In spite of the high variability observed, data generated by these experiments provide effective guidance on cometabolite and surfactant addition for in situ bioremediation in these test soils.

## **Static Cell Test Results**

### **Fabrication of test cells**

Results of this test indicated no significant sorption of any of the compounds by either polypropylene or teflon at 12 or 34 days (Table 9). While the initial trial test cells were constructed of teflon, polypropylene units were used for all subsequent testing, including the work conducted herein.

### **Environmental conditions within the test cells**

Each of the measurements made during flow of carbon dioxide-free air through the cells indicated that aerobic conditions were present in the intraparticle space of every cell during the entire 7-month incubation period.

The moisture dosing study indicated that 10 mL of fluid was insufficient to moisten the soil for readings with the probes, while 20 mL of fluid required several days for the moisture to reach the probe in the cells (Figure 11). Shaking did not improve the detection of the 10 mL spray at the moisture probe, and only marginally improved detection of moisture with the 20 mL spray. For this reason, 30 mL was selected as the appropriate volume for use in routine moistening of this soil. Initial incubations in the chambers indicated that with the fan on to circulate air through the chamber, moistening intervals of 5 to 7 days were needed to maintain moisture at the probe in the static cells. Therefore, moisture was added weekly in 30-mL increments.

### **Effects of treatment**

Numbers of microorganisms in the abiotic controls fell to zero following the addition of mercuric chloride (Figure 12). Except for the high nutrient treatment sample at 6 months, the growth patterns of total heterotrophs recovered on PTYG and microorganisms recovered on BSA-TNT media were similar in magnitude and behavior for both the low and high nutrient treatments. This result indicated that many, if not all, of the microorganisms recovered were the same on both media. Overall, microbial growth, as indicated by recovery on these media, was somewhat stronger during the first 2.5 months than during the remainder of the treatment period. The reasons for the

**Table 9**  
**Sorption of TNT and TNT Transformation Products by Centrifuge**  
**Bottles (Solution concentrations in polypropylene compared with**  
**teflon bottles after 12 and 34 days of incubation in the dark)**

Replicate No. and Analytical Chemistry Code <sup>1</sup>	Concentration of Compound, mg/L					
	TNT		2A4, 6DNT		4A2, 6DNT	
	Polypropylene	Teflon	Polypropylene	Teflon	Polypropylene	Teflon
<b>12 Days</b>						
1a	6.16	7.02	1.96	1.89	1.88	1.91
1b	6.19	7.15	1.95	2.09	1.81	2.05
1c	6.37	7.22	1.86	2.09	1.70	2.05
2a	5.76	5.85	2.05	1.94	1.91	2.00
2b	5.74	5.84	2.12	1.95	2.01	2.01
2c	5.46	5.92	2.14	1.87	1.99	1.86
3a	6.04	6.53	2.06	2.09	2.11	2.08
3b	5.88	6.55	1.90	2.00	1.91	2.01
3c	6.03	6.54	1.98	2.01	1.96	2.08
Mean	5.96	6.51	2.00	1.99	1.92	2.01
STD	0.25	0.49	0.09	0.08	0.11	0.07
<b>34 Days</b>						
1a	6.09	6.68	1.99	2.10	1.87	2.15
1b	6.17	6.65	2.00	2.04	1.87	1.96
1c	6.37	5.56	1.95	2.02	1.86	1.99
2a	6.16	4.90	2.02	1.94	2.03	1.91
2b	5.55	5.02	1.97	1.76	2.04	1.78
2c	5.45	4.91	1.98	1.79	1.82	1.83
3a	5.96	6.22	1.94	1.98	1.93	1.92
3b	6.02	6.33	1.93	2.12	2.01	2.10
3c	5.91	6.24	1.85	1.93	1.76	1.80
Mean	5.96	5.94	1.96	1.96	1.91	1.94
STD	0.27	0.69	0.04	0.11	0.09	0.12

<sup>1</sup> Replicates are designated 1, 2, and 3. Letters a, b, and c stand for analytical chemistry subsamples.

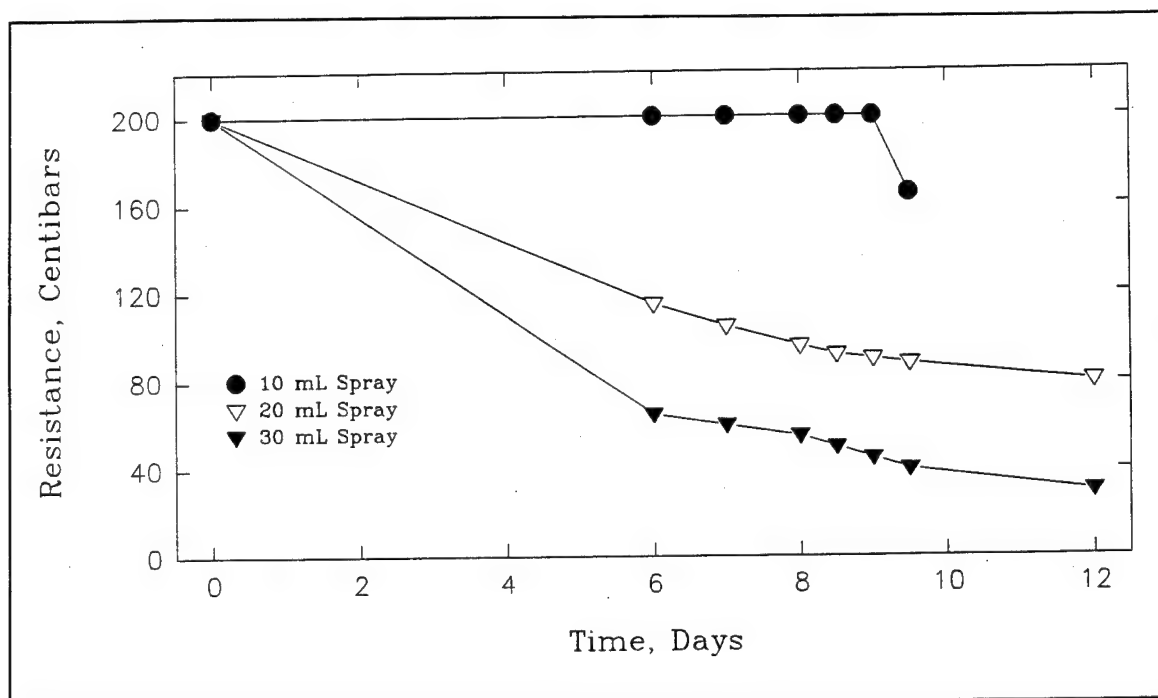


Figure 11. Time required for 10, 20, and 30 mL volumes of water to reach the moisture probe in the static cell following spray application (Resistance decreases as moisture approaches the probe.)

decline during the later portion of treatment were not determined, but may be related to the disappearance of TNT or the accumulation of toxic intermediates in the soils.

Over the first 2.5 months, the level of TNT dropped prodigiously in the high nutrient treatment, while remaining at approximately the initial level in the other three treatments (Figure 13). Over the course of 7 months, TNT levels in the high nutrient treatment dropped below 290 mg/kg, representing a disappearance of more than 97 percent of the original material. Levels in each of the remaining treatments fell to approximately half the original value over this same period. However, losses from the remaining treatments were not significant until after 4 months, by which time TNT in the high nutrient treatment had nearly disappeared. Losses of TNT from the low nutrient occurred slightly more rapidly than from the control treatments, indicating that active microbial populations were responsible for at least some of the observed removal. By months 6 and 7, no significant differences were evident between TNT levels in these three treatments, suggesting that much of the loss in the biotic control, and possibly some of the loss in the low nutrient control may have been abiotic in nature. By contrast, most of the 2ADNT- and 4ADNT-forming activity occurred in the treatments receiving nutrients and/or in the biological control. The abiotic control had little accumulation of 2ADNT and virtually no 4ADNT accumulation. Some DNT and trinitrobenzenes accumulated in the abiotic control, as well as the high nutrient treatment.

Log<sub>10</sub> Number of Microorganisms per Gram Dry Weight of Soil

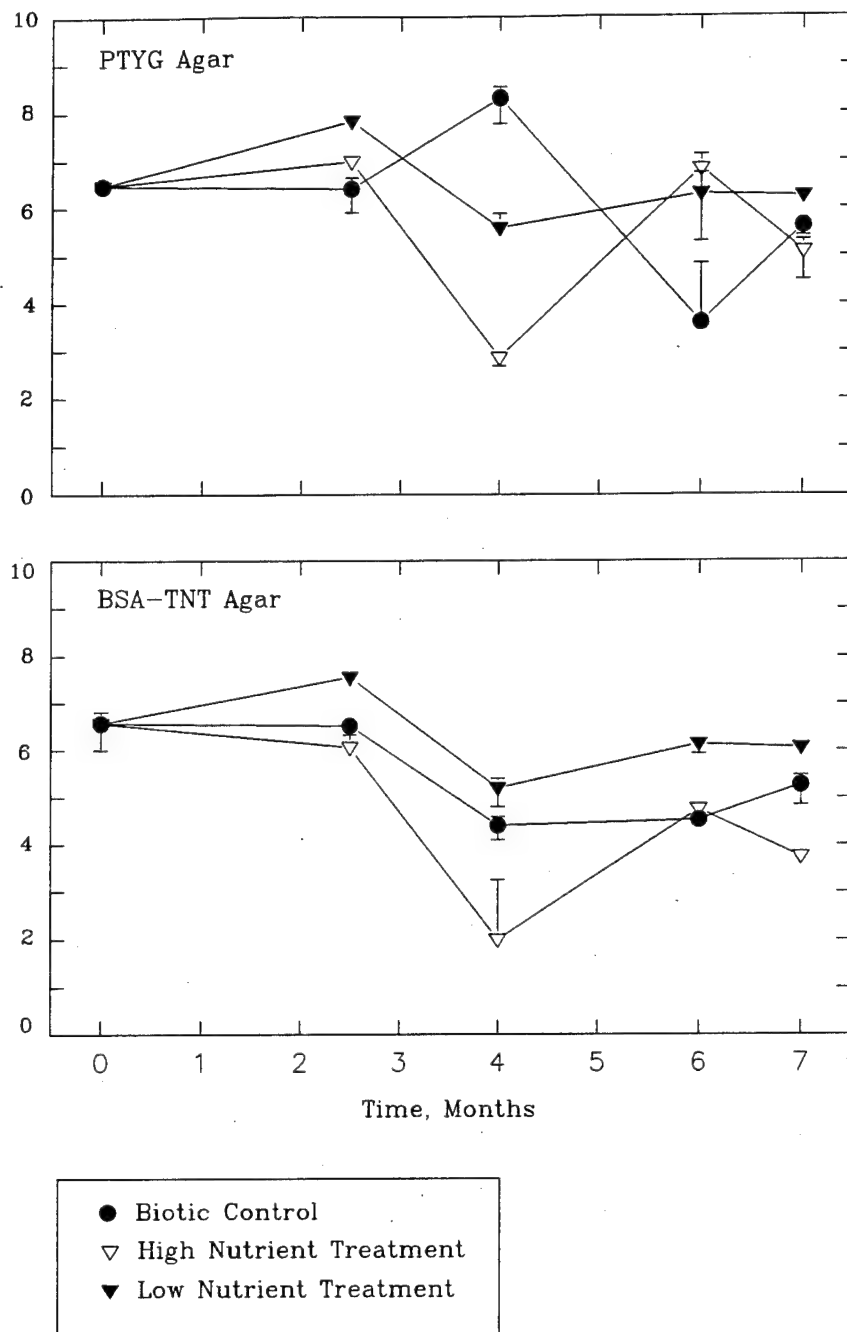


Figure 12. Total heterotrophic bacteria and microorganisms on BSA-TNT medium from the static cells during land-farming treatment of Hastings soil (No microorganisms were recovered from the abiotic controls. Values for the biotic controls are the means of 2 replicates  $\pm$  standard error of the mean, while those for the high and low nutrient treatments are the means of 4 replicates  $\pm$  standard error of the mean.)



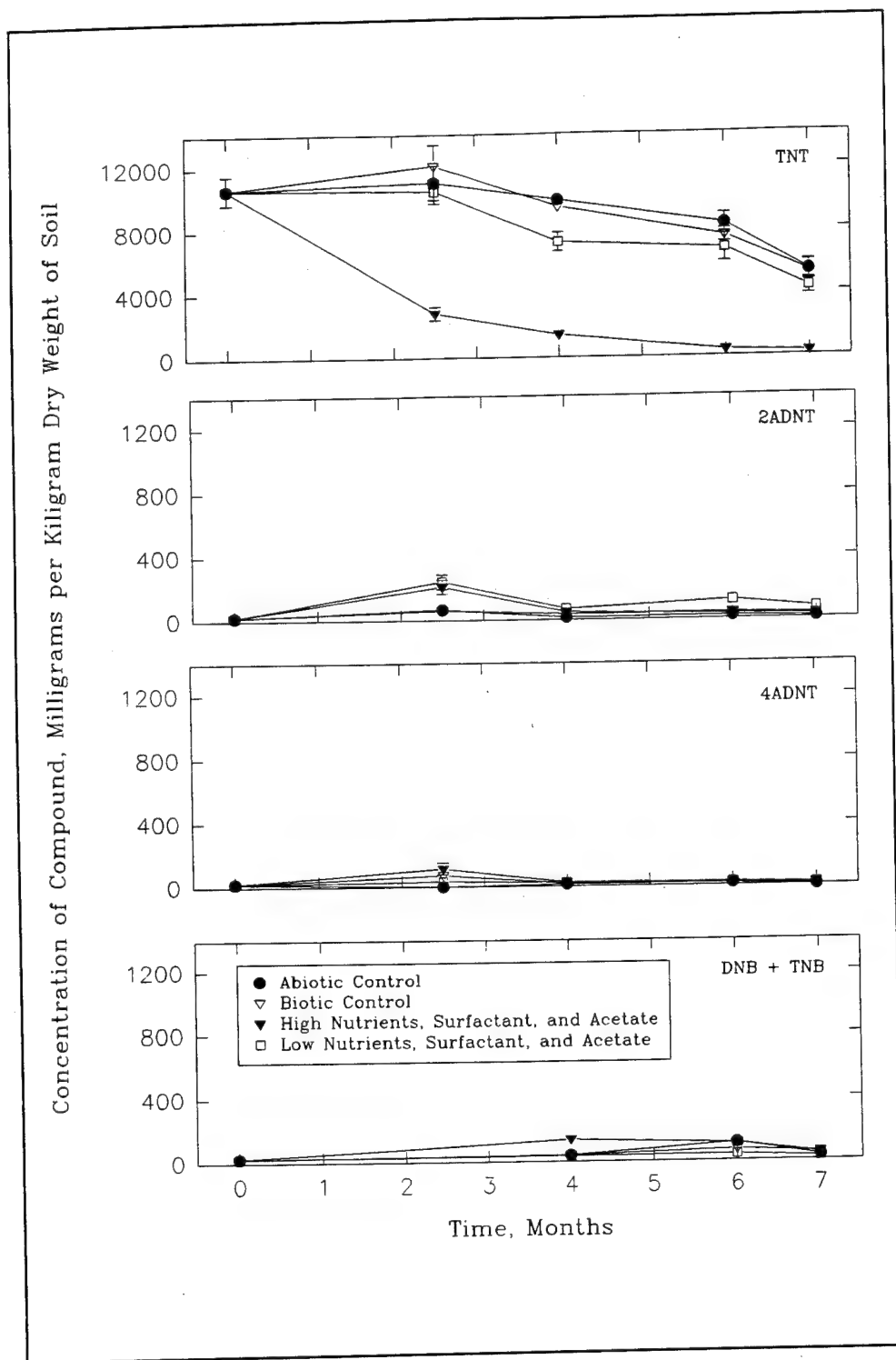


Figure 13. Changes in concentrations of TNT and TNT transformation products in the static cells during land-farming treatment of Hastings soil [Values given for the controls are the means of 2 replicates  $\pm$  standard error of the mean, while those for the low and high nutrient treatments are the means of 4 replicates  $\pm$  standard error of the mean (note y-axis scale difference).]

However, detectable levels of these materials were not found at 2.5 months of treatment.

The high nutrient plus acetate plus Tween 80 treatment produced the most extensive disappearance of TNT (approximately 2.2 percent remaining after 7 months), while also supporting some removal of 2ADNT and TNB (Figure 14). In contrast, the remaining treatments showed some loss of TNT towards the end of treatment, but none was as effective as the high nutrient plus acetate plus Tween 80 treatment.

When the initial and final soil samples were extracted and analyzed for azoxy compounds,  $18.35 \pm 2.200$  mg of the 2,2',6,6'-tetranitro-4,4'-azoxytoluene/kg DWE was found in the initial soil sample and  $448 \pm 258$  mg/kg DWE was present in the abiotic control. None of this azoxynitro-toluene was found in the biotic control or the low or high nutrient treatments. The 4,4',6,6'-tetranitro-2,2'-azoxytoluene was not present in the initial sample, the biotic control, and the low nutrient treatment. However,  $56.34 \pm 6.260$  mg/kg was present in the abiotic control, and  $13.99 \pm 0.300$  mg/kg DWE was present in the high nutrient treatment. No other peaks suggestive of other azoxy or related compounds were found in the vicinity of the standards.

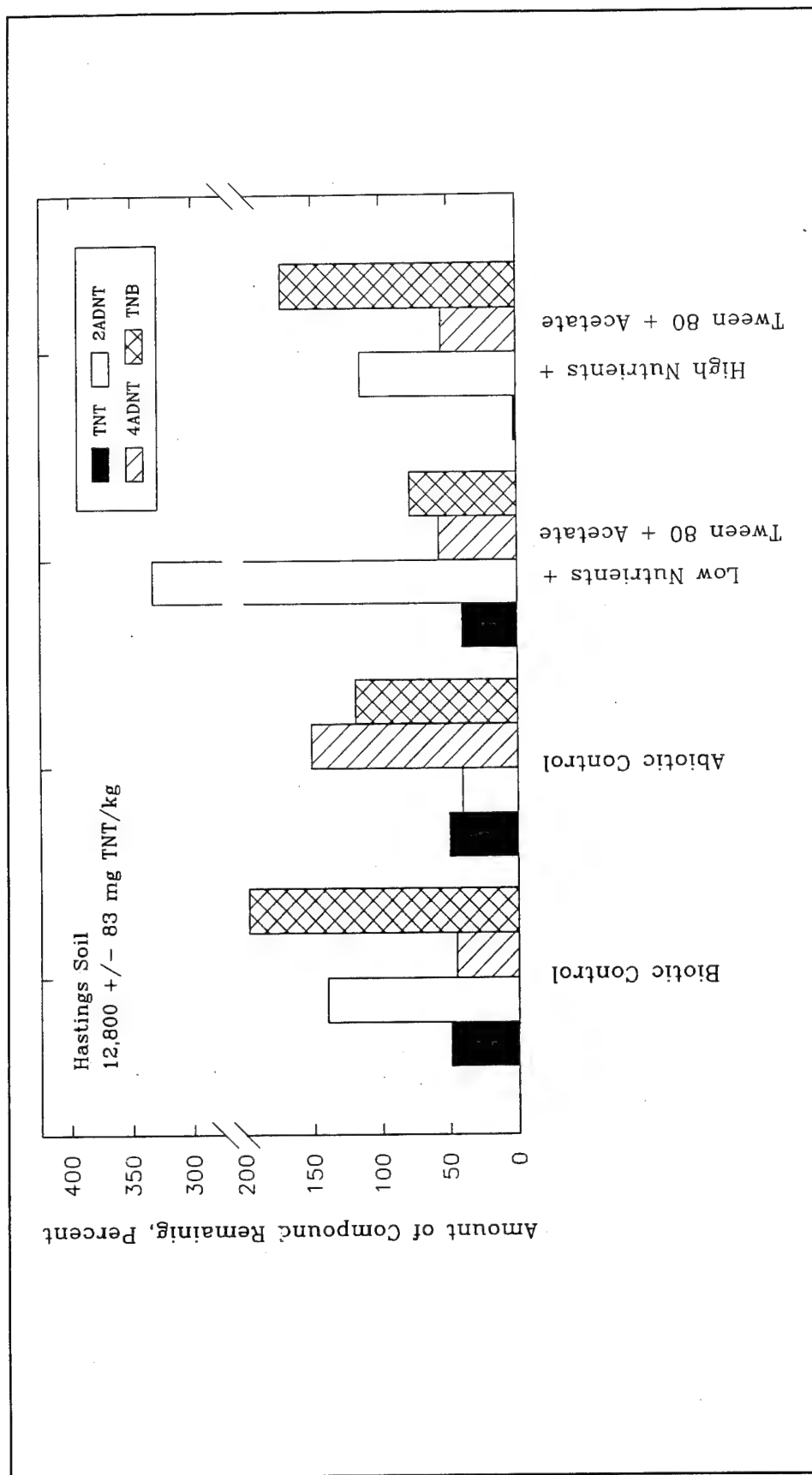


Figure 14. Comparison of accumulation or removal of TNT and TNT transformation products at 7 months of static cell treatment (Values given are the levels of each compound present at the end of treatment divided by the amount initially present. The number given under the name of the soil is the concentration of TNT present in the soil before treatment. No significant accumulations of dinitrotoluenes occurred in any of the treatments.)

## 4 Conclusions

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### Shake Flask Testing

In general, the addition of nutrients and toluene supported the treatment process. The addition of Tween 80 sometimes stimulated treatment. Each soil tested was unique in most of the properties examined, especially in the TNT degrading behavior of its microbial inhabitants. Replication of measurements within a soil sample were tight. The requirement for specific combinations of additives to accelerate treatment was site specific for the soil. The data support the need for individual testing at each site.

The variable requirement for a surfactant agrees with the findings of Pennington et al. (1995) who found that surfactants generally increased aqueous phase levels of the explosives, but the impact was less pronounced in soils having low explosives concentrations, as was the case here for Bangor and Crane soils. However, high concentrations of explosives in soil aqueous phases can be toxic or inhibitory to the degrading microorganisms. The present study indicated that the addition to a shake flask of a single charge of nutrients, cosubstrate, and a surfactant produced the complete disappearance of TNT and its transformation products from a soil having a low initial level of TNT. As the initial TNT levels increased, the amount of removal decreased. The weekly addition of nutrient, cosubstrate, and/or surfactant may ameliorate this effect to some extent; however, the upper limits of TNT concentration that preclude microbial activity must be determined. The soils tested in the present study did not have explosive concentrations high enough to preclude all microbial activity. However, the Weldon Springs soil which had a TNT level of approximately 42,000 mg/kg DWE (Pennington et al. 1995), exhibited no detectable microbial activity in the screening test.

Addition of the cosubstrates toluene and/or acetate sometimes inhibited growth slightly when used with nutrients. However, this inhibition was often offset when Tween 80 was used in combination with the cosubstrates. This may be related to the fact that Tween 80 is biodegradable and, therefore, a potential growth substrate.

The results of the present study indicate that in examining the feasibility of using biotreatment for clean up of explosives-contaminated soils, several

microbial requirements are important. These requirements include inorganic nutrients, cosubstrates, and sometimes a surfactant. Generalizing requirements for effective biotreatment at all sites is not good practice. Instead, requirements must be evaluated on a site-specific basis. Furthermore, processes used in land-farming treatment to solubilize explosives should be regulated to allow explosive degradation to keep pace with solubilization, precluding leaching of undegraded explosives into groundwater (Pennington et al. in press).

## **Static Cell Testing**

The results obtained from the static cell testing demonstrated that weekly dosing with 30 mL of the high nutrient solution produced the most rapid and complete disappearance of TNT. This corresponds to weekly dosages of 16.2 g of sodium acetate, 3.36 g of Tween 80, 0.304 g of ammonium nitrate, and 43.2 mg each of monobasic and dibasic potassium phosphate administered to 1 m<sup>2</sup> of soil to a depth of 1 cm over a 7 month period. The remaining treatments produced disappearances of TNT that were the same as or differed little from the abiotic control. The static cell test results indicated that the test system is useful for the evaluation of treatment of TNT contamination at the soil surface.

## **Implications for Engineering Treatability Studies**

Development of the shake and the static cell tests completes the tiered evaluation system (Figure 1). This system can now be used in treatability studies as a tool for determining the ability of the native microflora to degrade TNT, and to optimize microbial degradation in soil surface biotreatment. Information obtained from this system can be applied by the engineer in the design of pilot- or demonstration-scale systems.

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stimulated, but at other times inhibited, growth and treatment. Addition of the surfactant Tween 80 sometimes stimulated treatment. Addition of the cosubstrates toluene or acetate sometimes inhibited growth slightly when used with nutrients, but inhibition was commonly offset when Tween 80 was used in combination with the cosubstrates.

Treatment effectiveness and the required concentrations of additives were determined in the Tier III test—a static cell test developed specifically for this purpose. Results of static cell testing indicated that for Hastings soil, weekly dosing with a high nutrient solution produced rapid and nearly complete disappearance of TNT over a 7-month period.

Study results suggest that when examining the feasibility of explosives biotreatment using surface application of additives, site-specific factors operate to determine microbial requirements for inorganic nutrients and cosubstrates, as well as the possible need for a surfactant. Consequently, microbial requirements must be evaluated on a site-specific basis. Therefore, the tiered testing approach is ideally suited for optimizing treatment conditions prior to pilot-level tests.